REMARKS

With this response, claims 2, 3, and 19-21 have been amended, and new claims 26-37 have been added. Claims 4-18 and 24 have been cancelled, without prejudice or disclaimer. Thus, upon entry of this response, claims 2-3 and 19-23, and 25-37 will be pending.

Claim 2 has been amended to independent form, and to recite the subject matter of claim 24, now cancelled, without prejudice. This amendment is further supported by the specification at, *e.g.*, page 34, lines 2-6.

Claim 3 has been amended to recite that the L1 element comprises a sequence at least 95% similar to nucleotides 1-884 of SEQ ID NO:1. This is supported by the specification at, *e.g.*, page 11, lines 2-21, and page 34, lines 6-20.

Claims 19-21 have been amended to depend from claim 3.

New claim 26 and 32 recite that the L1 element comprises the sequence of residues 1-884 of SEQ ID NO:1. This is supported at, *e.g.*, page 11, lines 2-21. New claims 27 and 33 recite that the L1 element comprises SEQ ID NO:1. This is supported at, e.g., page 11, lines 10-11. New claims 28 and 34 recite that the region of the genome is within 5 million bases on either side of the SLE-associated marker. This is supported at, *e.g.*, page 47, lines 7-11. New claims 29 and 35 recite that the region of the genome is within 1.7 cM on either side of the SLE-associated marker. This is supported by, *e.g.*, Example 2 at page 58, lines 7-9. New claims 30 and 31 correspond to claims 22 and 23. Finally, new claims 36 and 37 are supported by the specification by, *e.g.*, Examples 1 and 2 (pages 57-58 of the specification).

Thus, upon entry of the present amendment, the independent claims will read as follows:

2. A method of identifying candidate genes involved in systemic lupus erythematosus (SLE), comprising
identifying a region of the genome adjacent to an SLE-associated marker, and

selecting any gene in the region having a distance between the first nucleotide of the gene and the first nucleotide of an L1 element of less than about 200,000 base pairs as a candidate gene involved in SLE,

wherein the L1 element comprises a sequence at least about 95% similar to the sequence of nucleotides 1-884 of SEQ ID NO:1.

3. A method of identifying candidate genes involved in SLE, comprising

identifying a region of the genome adjacent to an SLE-associated marker, and

selecting any gene in the region containing an L1 element in an intronic region or in a 5' or 3' regulatory region as a candidate gene involved in SLE,

wherein the L1 element comprises a sequence at least 95% similar to the sequence of nucleotides 1-884 of SEQ ID NO:1.

36. A method of identifying candidate genes involved in systemic lupus erythematosus (SLE), comprising

identifying a gene containing an L1 element in an intronic region or in a 5' or 3' regulatory region, and

selecting any gene within 1.7 cM of an SLE-associated marker as a candidate gene involved in SLE,

wherein the L1 element comprises a sequence at least 95% similar to the sequence of nucleotides 1-884 of SEQ ID NO:1.

No new matter has been added by way of this response. Each of the Examiner's objections and rejections are addressed below.

The Finality of the Office Action Should Be Withdrawn

The Examiner made the January 26, 2004 office action final on the alleged basis that Applicant's amendment to include the sequence of SEQ ID NO:1 and sequences having at least 95% sequence similarity to SEQ ID NO:1 necessitated new grounds of rejection, specifically a written description rejection (see page 10 of the office action).[1]

In response, the Examiner's attention is respectfully drawn to original claim 2, which recited an L1 sequence at least 95% similar to nucleotides 1-884 of SEQ ID NO:1. If the Examiner, for some reason, considers that this limitation is not supported by the specification in a manner complying with the written description requirement of 35 U.S.C. §112, this issue should have been

raised in the first office action. Thus, no claim amendment made in the response filed November 4, 2003 could necessitate any "new" grounds of rejection, and the Examiner cannot make the second office action final based upon claim limitations already examined prior to the issuance of the first office action. Accordingly, the finality of the January 26, 2004 office action should be withdrawn.

Claim Objections

The Examiner objects to claim 2 depending from claim 3, and requests correction to place claim 2 after claim 3. With this response, claim 2 has been amended to independent form, thus rendering this objection moot.

Enablement

The Examiner has rejected all claims as allegedly not complying with the enablement requirement. Specifically, the Examiner argues that "the Examiner is not aware of a working example that featured the method as applied to an affected, or predisposed patient to SLE and the concurrent discovery of the associated marker and L1" (Office Action, page 10, 1st paragraph). It is noted, however, that the Examiner acknowledges that the need for a mechanism [by which the present invention functions] is not required (Office Action, page 9-10, bridging sentence).

This rejection is respectfully traversed. First, it is noted that the Examples, particularly Examples 2 and 4, describe SLE susceptibility loci being found to be in proximity to L1 elements. To further establish that the invention is, in fact, enabled, the Examiner's attention is drawn to the following findings.

- A chromosomal locus statistically linked to SLE susceptibility and described in the specification harbor full-length L1 elements.
- A gene associated with those L1 elements has recently been shown to be abnormally expressed in SLE.

---

[1] It is noted that this rejection is traversed in the section entitled "Written Description".

- Treating SLE has recently been shown to lead to a reduction of expression of that gene product.

These observations, showing that the present disclosure provides adequate guidance as to the claimed method of identifying candidate genes involved in SLE, are discussed in more detail in the following sections.

The D4S403 Marker Region Contains Two L1 Elements. As described in Table 1 of the specification, D4S403, a marker associated with SLE susceptibility, is located on chromosome 4p. Initial analysis of this marker identified two L1 elements having at least 98% sequence identity to the reference L1 sequence. These L1 elements were located at approximately 0 and 3 million bases distance from the marker, respectively (see Table 1, 6th row at page 74; and claims 2, 3, and 36).

The D4S403 Region Contains the CD38 Gene. Specifically, the D4S403 marker region contains the gene encoding for CD38 antigen. Attached to this response are two entries from the UniSTS database at the NCBI PubMed website (www.ncbi.nlm.nih.gov/entrez/), the UniSTS database being a NCBI resource that reports information about markers, or Sequence Tagged Sites (STS). The first entry, attached at **Tab 1**, reports that D4S403 is located roughly at position 13.5 Mbp according to the SequenceMap. The second entry, attached at **Tab 2**, reports that the gene encoding the CD38 antigen is located on chromosome 4, roughly at position 15.6 Mbp according to the SequenceMap. Accordingly, the gene encoding the CD38 antigen is within 5 million base pairs from the D4S403 marker (see new claims 28 and 34).

The CD38 Gene Contains A Full-Length L1 Element. It has now been found that the intronic L1 element referred to in Table 1 as being located at approximately 3 million bases from the D4S403 SLE susceptibility marker is located in an intronic segment of the CD38 gene. This was discovered by a research group headed by Dr. Batzer, which group has published reports on the locations of L1 elements in the human genome. See, *e.g.*, Salem et al. (J Mol Biol 2003;326:1127-1146; attached at **Tab 3**). In supplementary data published on this group's website, http://batzerlab.lsu.edu, it is described that one L1 element, denoted "L1HS18" is located in the

gene having the Genbank accession number AC005798 (see line 18 of the table of Supplementary Data, attached at **Tab 4**). This accession number refers, in fact, to the CD38 gene (see page 2 of the UniSTS entry attached at Tab 2, describing AC005798 as describing the genomic sequence of CD38).

An Increased Proportion of B-cells in SLE Patients Are CD38^{+}. A higher proportion of B-cells express, or even express increased levels of, CD38 antigen in SLE patients. Specifically, Arce et al. (J Immunol 2001;167:2361-2369; **Tab 5**) has shown that the proportion of CD38 positive B-cells is significantly increased in SLE patients as compared to healthy adults and healthy children (see Arce et al., Figure 4 on page 2365, and accompanying text at page 2364, 2nd column, 1st full paragraph), and reports that "[m]ost SLE patients display a distinct population of CD20^{-}/CD19$^{+/low}$CD38^{++} blood cells" (page 2366, 1st column, 1st full paragraph), referring to Figure 7 on the following page. The reference also notes that "B-cells play a major role in the pathology of SLE in both human and murine SLE models, as they are responsible for the hypergamma-globulinemia and autoantibody production that characterize this disease" (page 2361, 1st column, 1st paragraph).

Anti-CD154 Antibody Treatment of SLE Patients Leads to Disappearance of CD38+ Cells. Finally, Grammer et al. (J Clin Invest 2003;112:1506-1520; **Tab 6**) reports that "before treatment, active-SLE patients had circulating CD38^{bright} Ig-secreting cells that were not found in normal individuals", and that "[d]isappearance of this plasma cell subset during treatment was associated with decreases in anti-double-stranded DNA … Ab levels, proteinuria, and SLE disease activity index" (Grammer et al., Abstract).

Accordingly, the specification provides adequate guidance to identify candidate SLE genes. In particular, the references cited above establish that proximity of an L1 element is predictive of a candidate SLE gene in a region neighboring an SLE-marker. This represents specific evidence of enablement given (1) the disclosure of the invention and (2) the level of skill in the art.

"To be enabling, the specification of the patent must teach those skilled in the art how to make and use the full scope of the claims of invention without 'undue experimentation.' " *Genentech Inc. v. Novo Nordisk*, A/S, 42 USPQ 2d 1001, 1004 (Fed. Cir. 1997) (quoting *In re Wright*, 27 USPQ 2d 1510, 1513 (Fed. Cir. 1993)). The court has held that a patent specification complies with a statute even if a "reasonable" amount of routine experimentation is required but such experimentation must not be "undue". *Enzo Biochem Inc. v. Calgene, Inc.* 52 USPQ 2d 1129, 1135 (Fed. Cir. 1999) (citing In re Wands, 8 USPQ 2d at 1404). The court in *Enzo Biochem* looked favorably on the factors set forth in *In re Wands* to consider in determining whether disclosure requires undue experimentation, which are:

(1) the quantity of experimentation necessary,
(2) the amount of direction of guidance presented,
(3) the presence or absence of working examples,
(4) the nature of the invention,
(5) the state of the prior art,
(6) the relative skill of those in the art,
(7) the predictability or unpredictability of the art, and
(8) the breadth of the claims.

Enzo Biochem, 52 USPQ 2d at 1135-1136 (quoting *In re Wands*, 8 USPQ 2d at 1404).

Applying the Wands factors to the facts set forth above to the claims as amended, Applicants submit that the specification enabled the claimed invention. In particular, the quantitative experimentation necessary to identify L1 elements in proximity to an SLE marker is not great, given the considerable advances in sequencing technology and genomic databases. Given that the present specification establishes a relationship between the L1 elements and candidate SLE genes, applying the presently claimed method to identify the candidate SLE genes was no more than routine.

The second factor is the amount of direction or guidance. The instant specification provides clear guidance for identifying candidate SLE genes (see, for example, page 32, line 32 to

page 34, line 27). This general guidance to use publicly available genomic information and computer programs such as BLAST to identify L1 elements was sufficient to successfully identify a candidate SLE gene, as shown by the above example with the D4S403 marker and CD38 gene.

Third, the present application does provide working examples. It is well-established, however, that "lack of working examples or lack of evidence that the claimed invention works as described should never be the sole reason for rejection the claimed invention on the grounds of lack of enablement" (MPEP section 2164.2). Additionally, the D4S403/CD38 example provided above does, in fact, show that the claimed invention works as described.

Fourth, the nature of the invention is one of a fairly routine and standard experimental or computerized system for analyzing genomic sequences.

Fifth, the state of the prior art is highly advanced. The sequencing of the human genome is finalized, L1 elements are known, a multitude of SLE markers have been identified, and the techniques for identifying additional SLE markers are well-developed (see, for example, the Gaffney et al., Moser et al., Tsao et al., and Tsao et al. references attached at Tabs 1-4 with the previously filed response to office action, submitted on November 4, 2003).

With respect to item six, the relative skill of those in the art paralleled the advanced state of the art. Thus, the skilled artisan would not have any problem scanning the genome for candidate SLE genes in proximity to SLE susceptibility loci comprising L1 elements.

With respect to item seven, the predictability of the claimed method, this has also been established by showing that the method could accurately predict the presence of a candidate gene in the region surrounding the disclosed SLE susceptibility D4S403 loci which is reported to contain L1 elements.

When considering the breadth of the claims, Applicants submit that they are fully enabled for the claimed scope in view of the details provided in the specification and Examples.

Further, the office action states that "[w]hile the cited art may in fact teach of the existence of markers well known in the art for SLE, the cited art is silent with respect to each of these known markers being associated with a L1 element of a certain % similarity located at a certain distance from the known marker" (page 10, 1st paragraph). However, the fact that the prior art teaches well-known SLE markers but not L1 elements of certain sequence similarities associated with candidate SLE genes merely underscores the novelty and non-obviousness of the present invention, and has no bearing on the enablement of the claims. Notably, the present specification enables the claims, as shown in the preceding paragraphs.

In view of the foregoing amendments and remarks, Applicants submit that the claims meet the enablement requirement, and the Examiner's rejection should be withdrawn.

Written Description

The Examiner has rejected claims 2, 3, and 19-25 as allegedly not complying with the written description requirement. Specifically, the Examiner argues that "the skilled artisan cannot envision the detailed chemical structure of the encompassed polynucleotides and/or proteins" and that "absent factual evidence, a percentage sequence similarity less than 100% is not deemed to reasonably support to one skilled in the art whether the biochemical activity of the claimed subject matter would be the same as that of such a similar known biomolecule" (Office Action, page 12, 1st paragraph; page 13, 2nd full paragraph).

This rejection is respectfully traversed. The present claims set forth a method of identifying candidate SLE genes, in which one step comprises selecting genomic L1 elements comprising a sequence having at least 95% sequence identity to residues 1-884 of SEQ ID NO:1. In this method, there is no need for the skilled artisan to "envision" each and every variant having a sequence identity within the claimed range, nor any need to compare its biochemical activity, because the present specification shows by experimental determination, *i.e.*, proof of practice, that L1 elements having the structural feature of a high sequence similarity to SEQ ID NO:1 can identify candidate SLE genes located adjacent to an SLE-associated marker (see, *e.g.*, Tables 1 and 3). This

identification can be made using BLAST or some similar algorithm on regions adjacent to an SLE marker, setting, if needed, certain sequence identities as selection criteria.

Further, it is noted that claims 22 and 30 call for an L1 element of at least 98% sequence identity to residues 1-884 of SEQ ID NO:1; that claims 23 and 31 call for an L1 element of at least 99% sequence identity to residues 1-884 of SEQ ID NO:1; that claims that claims 26 and 32 call for an L1 element comprising residues 1-884 of SEQ ID NO:1; and that claims 27 and 33 call for the L1 element comprising SEQ ID NO:1. Additionally, new claims 28 and 34, and new claims 29 and 35, call for the region of the genome being within 5 million bases and within 1.7 cM of the SLE susceptibility marker, respectively.

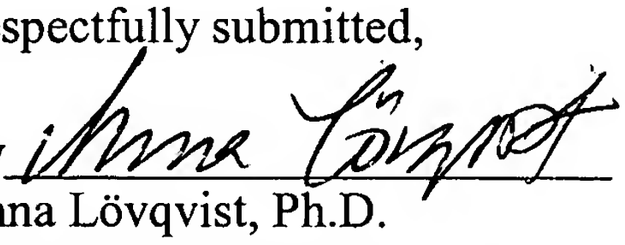
For these reasons, it is respectfully submitted that all claims comply with the written description requirement, and reconsideration and withdrawal of this rejection is earnestly requested.

Conclusion

In view of the above, each of the presently pending claims in this application is believed to be in condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

Dated: April 26, 2004

Respectfully submitted,

By 

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NCBI UniSTS

PubMed Entrez BLAST OMIM Taxonomy Structure

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Statistics
Related sites
e-PCR
Map Viewer
LocusLink
UniGene
dbSNP
GeneMap'99
RHdb
GDB
MGD
ZFIN
Genomic Biology

Bos taurus
Canis familiaris
Danio rerio
Homo sapiens
Mus musculus
R. novegicus
Sus scrofa

D4S403

UniSTS:15705

Primer Information

Forward primer:	**AGGTGGCCCTGAGTAGGAGT**
Reverse primer:	**TTTGAGGGAATGATTTGGGT**
PCR product size:	217-231 (bp), Homo sapiens
GenBank Accession:	Z16702

Homo sapiens

Name: D4S403

Also known as: 157XG3 AFM157xg3 HS157XG3

Cross References

LocusLink	LocusID:	9634
	Symbol:	STGD4
	Description:	Stargardt disease 4 (autosomal dominant)
	Position:	4p
LocusLink	LocusID:	64695
	Symbol:	SLEB3
	Description:	systemic lupus erythematosus susceptibility 3
	Position:	4p16-15.2
SNP	rs10682638, rs3138722, rs3219959	Summary
RH details	RH15122	Genebridge4
	RH28038	Genebridge4
	RH59461	Genebridge4
GDB	GDB:188106	

Mapping Information

View all results using the Map Viewer

D4S403	SequenceMap:	Chr 4	**mv**
	Position:	13501829-13502049 (bp)	
D4S403	deCODE Map:	Chr 4	**mv**
	Position:	26.71 (cM)	

D4S403	WI-YACMap:	Chr 4	**mv**
	Position:	34 (ordinal)	
	Reference Interval:	WC4.1	
D4S403	WI-RHMap:	Chr 4	**mv**
	Position:	80.6 (cR3000)	
	Lod score:	P0.01	
AFM157xg3	GenethonMap:	Chr 4	**mv**
	Position:	24.90 (cM)	
AFM157xg3	Marshfield Map:	Chr 4	**mv**
	Position:	25.90 (cM)	
AFM157xg3	GM99-GB4Map:	Chr 4	**mv**
	Position:	66.87 (cR3000)	
	Lod score:	F	
	Reference Interval:	D4S412-D4S1601 (3.7-28.2 cM)	

Electronic PCR results

Genomic (5)

Z16702.1	119 .. 345 H. sapiens (D4S403) DNA segment containing (CA) repeat; clone AFM157xg3; single read (345 bp)
AADD01043185.1	7916 .. 8142 Homo sapiens chromosome 4 WGSA:43184 contig 43184 scaffold 75, whole genome shotgun sequence (10078 bp)
AADB01025643.1	18236 .. 18462 Homo sapiens chromosome 4 WGA:25642 contig 25642 scaffold 1010, whole genome shotgun sequence (23384 bp)
AADC01038634.1	195032 .. 195258 Homo sapiens chromosome 4 CSA:38633 contig 38633 scaffold 9577, whole genome shotgun sequence (522571 bp)
AC007126.6	62034 .. 62254 Homo sapiens chromosome 4 clone C0496D08, complete sequence (218698 bp)

Questions or Comments?
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G33802

UniSTS:16981

Primer Information

Forward primer:	**AGTTTCTTCAGTGTGTGAAAAATCC**
Reverse primer:	**CAAAATCTTCAGCTCTGCACC**
PCR product size:	152 (bp), Homo sapiens
GenBank Accession:	G33802

Homo sapiens

Name:	G33802
Also known as:	SHGC-50808

Cross References

LocusLink	LocusID:	952
	Symbol:	CD38
	Description:	CD38 antigen (p45)
	Position:	4p15
UniGene	Hs.174944	CD38 antigen (p45)

Mapping Information

View all results using the Map Viewer

G33802	SequenceMap:	Chr 4	**mv**
	Position:	15601053-15601204 (bp)	

Electronic PCR results

RefSeq mRNA (1)

NM_001775.2	955 .. 1106 Homo sapiens CD38 antigen (p45) (CD38), mRNA (1494 bp)

mRNA (4)

D84276.1	951 .. 1102 Homo sapiens mRNA for CD38, complete cds (1408 bp)
D84277.1	727 .. 878 Homo sapiens mRNA for CD38 alt, complete cds (1036 bp)
BC007964.2	896 .. 1047 Homo sapiens CD38 antigen (p45), mRNA (cDNA clone MGC:14106

IMAGE:4309086), complete cds (1225 bp)

M34461.1 917 .. 1068 Human lymphocyte differentiation antigen CD38 mRNA, complete cds (1233 bp)

Genomic (5)

AC005798.10 92662 .. 92813 Homo sapiens chromosome 4 clone RP11-442P12, complete sequence (206631 bp)

D84284.1 272 .. 423 Human DNA for CD38, exon 8 and complete cds (1412 bp)

AADD01043317.1 4154 .. 4305 Homo sapiens chromosome 4 WGSA:43316 contig 43316 scaffold 75, whole genome shotgun sequence (15997 bp)

AADB01025721.1 9443 .. 9594 Homo sapiens chromosome 4 WGA:25720 contig 25720 scaffold 1015, whole genome shotgun sequence (67753 bp)

AADC01038832.1 56424 .. 56575 Homo sapiens chromosome 4 CSA:38831 contig 38831 scaffold 9716, whole genome shotgun sequence (212480 bp)

Working Draft phase 1 (from GenBank HTGS division) (1)

AC092459.2 107687 .. 107838 Homo sapiens chromosome 4 clone CTD-2185J18, *** SEQUENCING IN PROGRESS ***, 16 unordered pieces (170099 bp)

ESTs (5 of 10)[Show All Hits]

BF513368.1 172 .. 323 UI-H-BW1-amk-e-04-0-UI.s1 NCI_CGAP_Sub7 Homo sapiens cDNA clone IMAGE:3070182 3' (481 bp)

AA291308.1 16 .. 167 zs18f10.s1 NCI_CGAP_GCB1 Homo sapiens cDNA clone IMAGE:685579 3' similar to gb:M34461 LYMPHOCYTE DIFFERENTIATION ANTIGEN CD38 (HUMAN); (341 bp)

AA318583.1 87 .. 238 EST20678 Spleen I Homo sapiens cDNA 5' end similar to CD38 antigen (263 bp)

AA748192.1 68 .. 219 nx98g05.s1 NCI_CGAP_GCB1 Homo sapiens cDNA clone IMAGE:1270328 3' similar to gb:M34461 LYMPHOCYTE DIFFERENTIATION ANTIGEN CD38 (HUMAN); (312 bp)

AA765500.1 168 .. 323 oa07a09.s1 NCI_CGAP_GCB1 Homo sapiens cDNA clone IMAGE:1304248 3' similar to gb:M34461 LYMPHOCYTE DIFFERENTIATION ANTIGEN CD38 (HUMAN); (554 bp)

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LINE-1 preTa Elements in the Human Genome

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**Corresponding author*

The preTa subfamily of long interspersed elements (LINEs) is characterized by a three base-pair "ACG" sequence in the 3′ untranslated region, contains approximately 400 members in the human genome, and has low level of nucleotide divergence with an estimated average age of 2.34 million years old suggesting that expansion of the L1 preTa subfamily occurred just after the divergence of humans and African apes. We have identified 362 preTa L1 elements from the draft human genomic sequence, investigated the genomic characteristics of preTa L1 insertions, and screened individual elements across diverse human populations and various non-human primate species using polymerase chain reaction (PCR) assays to determine the phylogenetic origin and levels of human genomic diversity associated with the L1 elements. All of the preTa L1 elements analyzed by PCR were absent from the orthologous positions in non-human primate genomes with 33 (14%) of the L1 elements being polymorphic with respect to insertion presence or absence in the human genome. The newly identified L1 insertion polymorphisms will prove useful as identical by descent genetic markers for the study of human population genetics. We provide evidence that preTa L1 elements show an integration site preference for genomic regions with low GC content. Computational analysis of the preTa L1 elements revealed that 29% of the elements amenable to complete sequence analysis have apparently escaped 5′ truncation and are essentially full-length (approximately 6 kb). In all, 29 have two intact open reading frames and may be capable of retrotransposition.

Keywords: retrotransposon; insertion polymorphism; mobile elements

Introduction

Computational analysis of the draft sequence of the human genome indicates that repetitive sequences comprise 45–50% of the human genome mass, 17% of which consists of long interspersed elements (LINE-1s or L1s).[1–3] L1 elements are restricted to mammals, having expanded as a repeated DNA sequence family over the last 150 million years.[4] Full-length L1 elements are approximately 6 kb long and propagate *via* an RNA intermediate in a process known as retrotransposition. L1 retrotransposition likely occurs by a mechanism termed target primed reverse transcription (TPRT).[5] This mechanism of mobilization provides two useful landmarks for the identification of young L1 inserts: an endonuclease related cleavage site[6–8] and direct repeats or target site duplications flanking newly integrated elements.[9]

L1 retrotransposons have had a significant impact on the human genome through a variety of different mechanisms. *De novo* insertions disrupting open reading frames and splice sites have resulted in a number of human diseases,[10–12] new L1 integrations have been shown to have the potential to alter gene expression,[13,14] and once in the genome L1 elements provide regions of sequence identity blanketing the genome, that can be exploited during recombination.[15] L1 elements also generate sequence duplications by transducing adjacent genomic sequences at their 3′ end, thereby "shuffling" genomic sequence.[16–18] More recently, it has been suggested that L1 elements have paradoxical roles in genomic stability by serving both as molecular band aids, repairing double-stranded breaks in mammalian cells and

† These authors contributed equally to this work.

Abbreviations used: LINE, long interspersed elements; L1, LINE-1; Ta, transcribed, subset a.

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as suspects for the generation of genomic deletions.[19–21] Thus, L1 elements exert a significant influence on the architecture of the human genome and provide dynamic units capable of ongoing change.

As a result of the limited amplification potential of the diverse L1 gene family, a series of discrete L1 subfamilies exist within the human genome.[4,22] L1 elements have expanded at different times during mammalian evolution, producing subfamilies of various ages.[4,22] Depending on the amplification period of the L1 subfamily, some L1 elements may be unique to a single lineage, species, or even a single population. Such is the case with the L1Hs (human specific) Ta (transcribed, subset a)[23] subfamily, which has been shown to be present only in the human lineage.[24]

Even though there are approximately 500,000 L1 elements in the human genome only a limited subset of 30–60 L1 elements appear to be capable of retrotransposition.[25,26] *De novo* L1 insertions resulting in human disease are largely a product of L1Hs Ta integrations, which have been shown to be the youngest most active L1 subfamily found in the human genome.[24,27,28] However, at least one L1 insert (JH-28) in exon 14 of the factor VIII gene resulting in hemophilia A, was the result of a preTa insertion, providing the first proof that preTa L1 elements are also currently capable of retrotransposition.[12] Previous studies have shown that some members of the preTa L1 subfamily have inserted so recently in the human genome that they are polymorphic with respect to insertion presence/absence,[27,29] all of which makes preTa L1 elements a likely source of identical-by-descent mobile element-based variation for the study of human population genetics.

Members of the L1 preTa subfamily share a common three base-pair diagnostic sequence within the 3′ untranslated region (UTR), which separates them from the other L1 subfamilies. As the name suggests, the preTa L1 subfamily is believed to predate the amplification of the L1Hs Ta subfamily in the human lineage. However, the phylogenetic origin and level of human genetic diversity associated with preTa L1 elements remains largely undefined. Here, we report a comprehensive analysis of the preTa L1 subfamily from the draft sequence of the human genome.

Results

L1 preTa subfamily copy number

To identify recently integrated preTa subfamily L1 elements from the human genome, we searched the draft sequence of the human genome (database version: BLASTN 2.2.1 (Apr-13-2001)) using the Basic Local Alignment Search Tool (BLAST)[30] with an oligonucleotide sequence that is complementary to a highly conserved motif in the 3′ untranslated region (UTR) of preTa L1 elements. This 19 base-pair (bp) query sequence (CCTAATGCTAGATGA CACG) includes the preTa subfamily-specific diagnostic mutation "ACG" at its 3′ end (position 5930–5932 relative to LRE-1).[31] We identified 362 unique preTa L1 elements from 2.868×10^9 bp of available human draft sequence. Extrapolating this number to the actual size of the human genome (3.162×10^9 bp), we estimate that this subfamily contains about 400 elements. Taken with the estimate from the L1Hs Ta data,[24] we estimate that there are over 900 human specific LINE-1 elements in the human genome. Of the 362 preTa L1 elements retrieved, six resided at the end of sequence contigs and were not amenable to additional analysis. Of the 356 (362 − 6) remaining elements, 105 (26%) were essentially full length, and 251 were truncated to variable lengths. Alignment and sequence analysis of the full-length elements revealed that 29 contained two intact open reading frames and therefore may be capable of retrotransposition. The complete data set is available on our web site† (under publications).

Estimated subfamily age

The average ages of L1 elements can be determined by the level of sequence divergence from the subfamily consensus sequence using a neutral mutation rate for primate non-coding sequence of 0.15% per million years.[32] The mutation rate is known to be about ten times greater for CpG bases as compared to non-CpG bases as a result of the spontaneous deamination of 5-methyl cytosine.[33] Thus, two age estimates based upon CpG and non-CpG mutations can be calculated for the preTa subfamily of L1 elements. A total of 74,048 bases from the 3′ UTR of 356 preTa L1 elements were analyzed. In all, 361 nucleotide substitutions were observed. Of these, 303 were classified as non-CpG mutations against the backdrop of 71,912 total non-CpG bases, producing a non-CpG mutation density of 0.004213 (303/71,912). Based upon the non-CpG mutation density and a neutral rate of evolution (0.004213/0.0015), the average age of the L1 preTa LINE-1 elements was 2.81 million years old. A total of 58 CpG mutations out of 2136 total CpG nucleotides were found across the same 356 LINE elements, yielding a CpG-based mutation density of 0.027154 (58/2,136). With the expectation that the CpG mutation rate is about tenfold higher than the non-CpG mutation rate, the approximate age of the L1 preTa subfamily using the CpG mutation density is 1.86 million years old. These estimates are in good agreement with one another and taken together, these estimates produce an average age of 2.34 million years old, which is in good agreement with the idea that the preTa L1 subfamily is evolutionarily older than the L1Hs Ta subfamily (estimated average age 1.99 million years).[24,27] In

† http://batzerlab.lsu.edu

addition the average age estimates reported here provide a relative time frame by which to compare L1 retrotransposition activity, and should not be confused with the age of origin.

Similar to the L1Hs Ta subfamily, the preTa L1 subfamily can also be grouped into two subgroups, ACG/A and ACG/G, based on an "A" or "G" base at position 6015 relative to L1.2 (accession number M80343). In order to determine the relative ages of each subgroup, we analyzed the level of sequence divergence in each subgroup. The ACG/A subgroup contained 127 total nucleotide substitutions, with 98 of these classified as non-CpG mutations against the backdrop of 20,402 total non-CpG bases. This yields a non-CpG mutation density of 0.004803 (98/20,402) and produces an estimated age of 3.20 million years old. Of 127 total mutations, 29 were classified as CpG mutations against a backdrop of 606 CpG total bases, which yields a CpG mutation density of 0.047855 (29/606) producing an estimated age of 3.28 million years. The ACG/G subgroup contained 221 total nucleotide substitutions with 191 of these classified as non-CpG mutations against the backdrop of 51,106 total non-CpG bases, which yields a non-CpG mutation density of 0.003737 (191/51,106), producing an estimated age of 2.49 million years old. Of 121 total mutations, 30 were classified as CpG mutations against a backdrop of 1518 CpG total bases, which yields a CpG mutation density of 0.019763 (30/1518) producing an estimated age of 1.35 million years. We calculated the average age of each subgroup as 1.92 and 3.24 million years for the ACG/G and ACG/A, respectively. Although it is likely that the L1Hs Ta subfamily is derived from one of the preTa L1 subsets based on the estimated ages of these L1 subfamilies, the transition intermediates between preTa and Ta subfamilies are not clear.

Table 1. PreTa L1 integration sites

PreTa L1 integration sites	Number
TTTT/A	60
TCTT/A	37
CTTT/A	20
TTTA/A	18
TTTC/A	18
TTTT/G	16
TTCT/A	14
TCTT/G	7
CTTT/G	5
ATTT/A	5
CTTT/C	5
TTTT/C	4
TGTT/A	3
TATT/A	3
TATT/G	3
TCTT/C	2
TTTC/C	2
TCTC/A	2
GTTT/A	2
ATTT/C	2
GCTT/T,TTTT/T,TTTG/A,TTTC/T,TTTC/T,TTGT/G, TTAT/A,TGAT/G,TCTT/T,TCAT/A,TATC/A,TATA/T, TAAA/C,GCTT/A,CCTT/A,CATT/G,CATT/A,ACTT/G, ACTT/A,ACTA/C,ACCT/A,ACAC/T,ACAA/A,AAAA/A	1 each

Features of L1 preTa integration sites

One hallmark of L1 integration is the generation of target site duplications flanking newly integrated elements. Two thousand bases of flanking sequence on each side of the element were searched for target site duplications. Clear target site duplications are considered to be target site duplications at least ten bases in length. Of the 356 elements analyzed, we were able to identify clear target site duplications for 252 elements. We then determined the integration sites for these 252 preTa L1 insertions with clear target duplications. A complete list of L1 integration sites is shown in Table 1, and further supports the notion that some integration sites are more common than others.[6,7,34]

A large number of preTa L1 elements had no observable target duplication sites. One possible explanation for this observation is that these elements have relatively short target site duplications. Alternatively, these elements may represent forward gene conversion events of older pre-existing L1 elements that by mutation, have rendered their target site duplications unrecognizable. Some of these events may also represent integrations that have occurred independent of endonuclease cleavage, that has previously been proposed as a mechanism for the repair of doubled-stranded breaks in DNA.[35–37]

To further characterize the preTa L1 insertions, we determined the DNA base content for sequence blocks 1 kb and 2 kb flanking all preTa L1 insertion sites with target site duplications of at least 10 bp. Flanking sequence was then grouped according to GC content with only data for the 1 kb sequence blocks shown in Figure 1. Our data suggest that preTa L1 elements integrate preferentially in genomic regions with GC content less than 36%, but are present in genomic regions with GC content as low as 26% and as high as 52%. A similar insertion site preference was observed for 2 kb sequence blocks as well as for the previously reported L1 Ta subfamily[24] and other L1 subfamilies.[38] In addition, we also analyzed preTa L1 elements inserted in repetitive sequences and

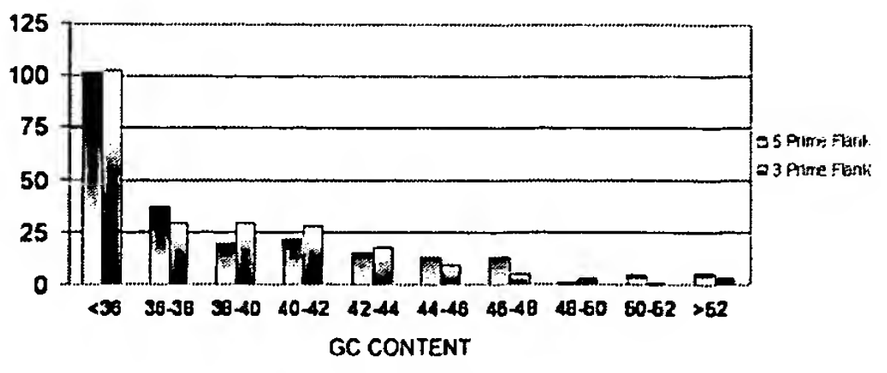

Figure 1. Analysis of preTa L1 pre-integration sites. GC content was calculated for L1 insertion flanking sequences of 1 kb and 2 kb. The 1 kb results are shown here.

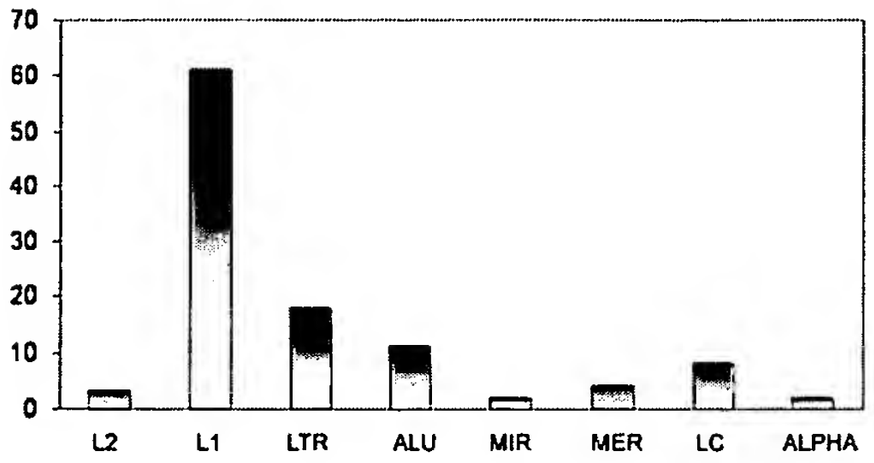

Figure 2. PreTa L1 integrations within other repetitive elements. PreTa L1 insertions within mobile elements were grouped according to the element in which they inserted. Mobile element categories include LINE-2 (L2), LINE-1 (L1), long terminal repeats (LTR), *Alu* (ALU), mammalian-wide interspersed repeats (MIR), medium reiteration frequency sequences (MER), low complexity sequence (LC), Alphoid satellite repeats (ALPHA).

grouped them according to the repeat family in which they reside (Figure 2). This analysis showed that preTa L1 elements insert most frequently in other L1 elements, which is expected both because L1 sequences occupy a large percentage of the human genome and because L1 elements are less GC-rich relative to other mobile element families, such as *Alu* elements, making them more susceptible to subsequent L1 integrations. Lastly, preTa L1 containing regions were analyzed to determine the distance from the integration to the nearest gene. A total of 12 preTa L1 elements reside within 25 kb of novel or known genes as denoted by GenBank annotation, including one full length preTa element, L1AD242, which inserted into intron 23-24 of the retinoblastoma susceptibility protein 1 gene and accounts for 6072 bp of the 7988 bp intron.

Sequence diversity

PreTa L1 sequence diversity is also created by variable 5′ truncation with some of the elements in the human genome only a few hundred base-pairs in length, whereas some full-length elements are over 6000 base-pairs. This phenomenon is classically attributed to the lack of processivity of the reverse transcriptase enzyme in the creation of the L1 cDNA. The point of truncation is traditionally believed to occur as a function of length, where shorter inserts are more likely to occur in the human genome than longer elements.[39] Our data show that there is an enrichment of full-length elements in the human genome, and like the Ta L1 elements many preTa L1 elements have been faithfully replicated in their entirety and inserted into new genomic locations. Of the 356 elements examined (362 total minus six elements located at the end of sequencing contigs), 97 were over 6000 base-pairs long, representing a much larger preTa L1 size class than any other size class (Figure 3). By contrast, very few elements were found in the size ranges between 4000 and 5500 bases, with only 14 of the 356 elements truncated to this particular size range. A bimodal distribution in the size of the elements is created, since there are a significant number of preTa L1 elements that are severely 5 prime truncated and those that are full-length with the average preTa element length of roughly 2700 bp and the median preTa element

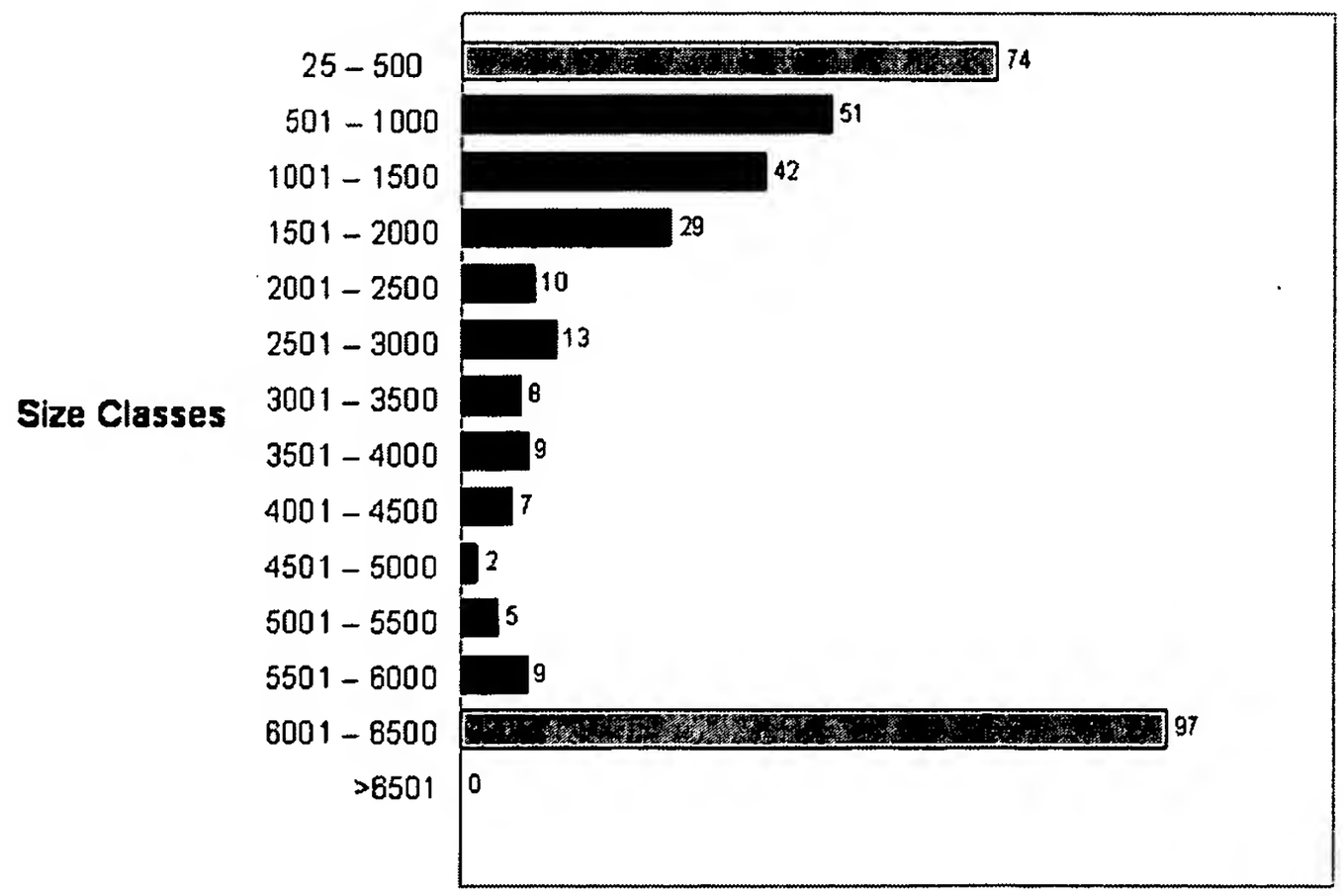

Figure 3. PreTa L1 element genomic size distribution. The following schematic shows the size distribution of preTa L1 elements. Elements are grouped in 500 bp intervals ranging from 25 bp in length to >6501 bp in length. The two most common size intervals are denoted in gray.

Table 2. Summary of preTa L1 analysis

Loci analyzed by PCR	254
Fixed present	200
High frequency insertion polymorphisms	11
Intermediate frequency insertion polymorphisms.	22
Low frequency insertion polymorphisms	0
Total preTa insertion polymorphisms	33
Inserted in paralogous sequences	3
No pre-integration site amplified in primates	9
No PCR results	9
Loci not analyzed by PCR	
L1 elements inserted in other repeats	102
End of contig	6
Total preTa L1 elements analyzed	362

length of roughly 1600 bp. A total of 196 elements were small, with sizes less than 2000 bp, with 125 of these only 50–1000 bases in length. In addition 28% (100/356) of the preTa L1 elements examined were inverted at their 5 prime end, which is believed to occur by an event known as twin priming where target-primed reverse transcription is interrupted by a second internal priming event, resulting in an inversion of the 5 prime end of the newly integrated LINE element.[40] Although L1 truncation is most likely the result of the relatively low processivity of the L1 reverse transcriptase, processes that form secondary structures in the RNA or DNA strands present at the integration site, like twin priming, may also be associated with L1 truncation. One expectation of this model is that a common truncation point should exist for L1 preTa elements. However, from our data we were not able to identify any common truncation points.

Similar to other L1 elements, preTa L1 elements exhibit a significant amount of sequence diversity in the 3 prime tails. In general, the 3 prime tails found in this L1 subfamily range in size from 4 bp to over 1600 bp in length. In all 64% contain AT-rich low complexity sequence, 13% have homopolymeric A tails with an average tail length of 15 bp, 6% have simple sequence repeats with the most common repeat family $TAAA_n$, and 17% contain complex sequence likely resulting from 3 prime transduction events. Three-prime transduction by L1 elements is a unique duplication event that occurs when an L1 sequence is transcribed

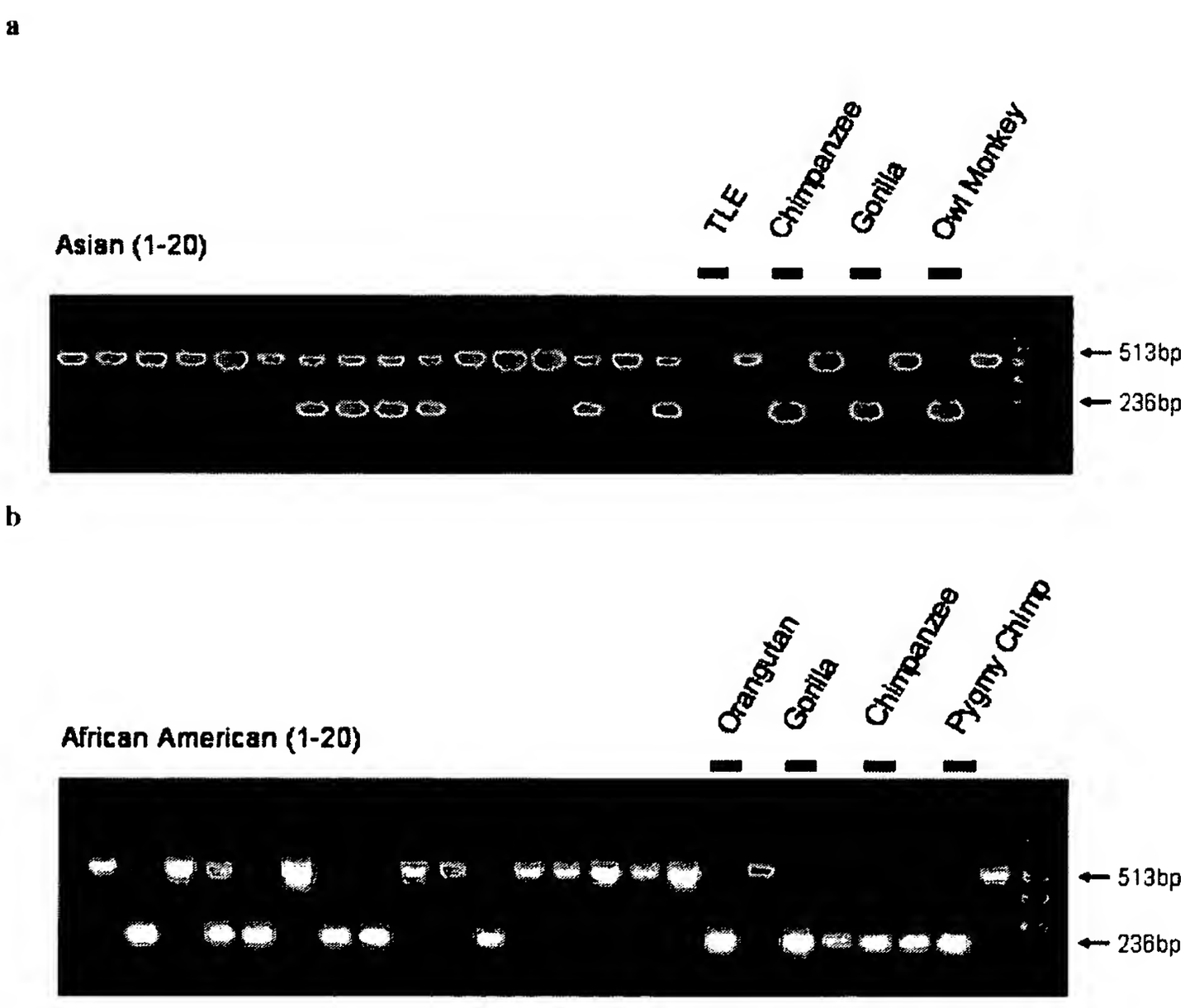

Figure 4. PreTa L1 insertion polymorphisms. This Figure is an agarose gel chromatograph of the PCR products from a survey of the human genomic variation associated with L1AD125. Amplification of the pre-integration site of this locus generates a 236 bp PCR product. Amplification of a filled site generates a 513 bp product (using flanking unique sequence primers). In this survey of human genomic variation 20 individuals from each of four diverse populations were assayed for the presence or absence of the L1 element, with Asian samples shown in (a) and African Americans shown in (b). The control samples are denoted by the black lines and were TLE buffer (10 mM Tris–HCl, 0.1 mM EDTA), common chimpanzee, pygmy chimpanzee, gorilla, orangutan and owl monkey DNA templates. In addition, this particular L1 element was absent from the genomes of non-human primates.

Table 3. PreTa L1 primers, PCR conditions, and associated human genomic diversity

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD1	AC080166.6	2	AATTCGCTGCATAATTTCTT	AAACATATGGCCATCTTGAC	FP	55	60	6835	249	578
L1AD2	AC090955.2	3	TTTTCTCCATGACTTGAGATGGT	TGCAATCATGAAAACCAGTG	FP	60	60	6308	245	265
L1AD3	AC018878.8	2	TGCACATGGATGTGTAAGAATAC	TTCTTCCCATAAGCATTGGT	FP	60	60	6448	339	245
L1AD4	AC053545.5	4	TTGATGCATTTCTGCATAAGG	CCAAGATTTTGGCTAGCATTT	FP	55	60	4528	295	188
L1AD5	AC079801.2	16	TCATCTCACAGAGCTCACAG	CTAGGAATCCTTCTGTCTGG	NP		60	749	326	150
L1AD6	AC073647.9	7	GCAAACACTGGTTCAAGAAG	TGGAGATAGTGTAGGCACAG	FP	55	60	1741	87	233
L1AD7	AC093607.3	4	Inserted in repeats		R					
L1AD8	AC079926.7	4	GCCTCTTTCTTAGTCAAGCA	AGGTCACAAGGGACATTTCT	NP		60	857	417	208
L1AD9	AC012593.8	2	CAGGTAGGGGAAAGGAGGAG	TGGGCTTATTATCCCCTTGA	FP	55	60	1034	392	342
L1AD10	AC016906.7	2	TGTATTTACCGGGGATGAGG	GCTGTCCCAAATTTCCAGAG	IF	60	60	3602	172	229
L1AD11	AC018465.8	2	GCACCTTGCTATTTGTTTTCT	CCCTAGAGCAATCACCAAAGA	FP	60	60	6515	458	185
L1AD12	AC083950.4	2	GGATAGGCAATGTGTTAGGT	TGCAGAGGCAGTTGTAACAT	FP	55	60	1106	603	303
L1AD13	AC097484.3	4	AAACCTATACATAGAAAATTGCTG	ACCCAGAACAAATGAACACT	FP	60	55	1368	473	424
L1AD14	AC012665.8	2	TTCTGCAACTATAGCCGTAA	ACAACAGACACAGAAGCAAA	IF	60	55	6187	136	173
L1AD15	AC093584.3	4	Inserted in repeats		R					
L1AD16	NG_000004.1	UNK	GGTTGAGAACCACTGTCATAA	GCCAGTGCTTAGATTTACCA	FP	60	60	6213	145	260
L1AD17	AC105459.1	7	ATTCCCCATTTTACGATTTT	GCTACTGCCGTGTTTTACA	FP	55	60	440	276	309
L1AD18	AC096764.3	2	AGATGCCCGGTCTACTACTT	AGCACTTTAAAGGCATCAAC	FP	55	60	3467	151	249
L1AD19	AC009156.9	16	ATATTGGCCAAAGCCTCTTA	TGGCAAGTCCTGAATGATAA	IF	55	55	3974	88	191
L1AD20	AC009156.9	16	CATTAGCAAGCTGATTCAAA	CTTTTGCCATGATTAGTGGT	HF	55	60	474	147	205
L1AD21	AC097522.4	4	CAGAAAGTCATCTCATCTTCC	TAAAGCATTCGTTGTTGTTG	FP	55	60	6528	353	587
L1AD22	AC092570.3	2	CCTCCTCACCTCCTTTTAAT	ATGAAGGGAAACGAGAAAAG	FP	55	60	562	63	220
L1AD23	AC018673.4	12	Inserted in repeats		R					
L1AD24	AC097451.2	4	TCGTTCCTCATCTCTTTGTT	AGCAAAAGCAGTCACTTTTC	FP	55	55	3467	382	396
L1AD25	AC023154.5	4	Inserted in repeats		R					
L1AD26	AC096769.3	4	TTGAGTTTTCCCTCCATGAAA	TCTGATGAATTGTGCCTGACA	FP	60	60	381	157	263
L1AD27	AC093877.3	4	AATATTTAACATGGCCCATAA	GGCATTGGTGTCAATGAGAA	FP	60	60	1171	110	834
L1AD28	AC096749.2	4	GAAGGCTTTATACTCCTTCTTGGA	TCATGGGAGATTTTTCAACTTTC	FP	55	60	6459	419	330
L1AD29	AC105150.2	8	GGACAGAAATACTGGCATCT	CACAATCTTATCTCAAGGGAAT	FP	60	60	6398	318	354
L1AD30	AC055820.7	18	CTTGATGGCAATACAGCCTAA	CCATTAATGTGGGCTCATAATCT	FP	60	60	1855	78	208
L1AD31	AC018626.8	18	GGGAAACGACAGAAGATGGA	GAATTTTGATTTGTGGGCATA	FP	60	60	1143	209	204
L1AD32	AC091613.3	1	End of contig		EC					
L1AD33	AC092798.3	3	Inserted in repeats		R					
L1AD34	AC012642.5	5	GGCTTGTGCTACACAGAGTT	CCAACCAGGAACAATAAAAG	FP	55	55	2816	519	247
L1AD35	AC021538.8	UNK	AAATGCCCACAAAATTCCTG	CCATGGGAGCTACTGGAAAA	FP	55	60	984	386	479
L1AD36	XM_037013.1	UNK	End of contig		EC					
L1AD37	AC099515.2	5	Inserted in repeats		R					
L1AD38	AC026703.4	5	CCCAGTTCTCCAAAATATCA	CACTTGCCTATGGTTCATTT	FP	55	55	5984	240	468
L1AD39	AC078857.12	3	Inserted in repeats		R					
L1AD40	AC078857.12	3	TCGTGACCTTATTAGCCACT	CCTCCATTTGCTACCTAGAG	FP	60	60	1680	512	633
L1AD41	AC078857.12	3	TGTTATTTCAGCTTTAACCATCAA	TTTAAAAATCAAGTATGGGAAAAA	FP	55	55	1202	141	242
L1AD42	AC093515.3	16	Inserted in repeats		R					
L1AD43	AC011597.27	3	Inserted in repeats		R					

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD44	AC079943.18	3	ATGCCATCCCCTGGATTT	TGGTTGCTCCAAAGGAACTT	NP		60	6591	530	316
L1AD45	AC061710.16	3	GAGCAAATTTGTCAGACAGAACA	TGGGATGGTTGAAATCAAATG	FP	60	60	3854	147	199
L1AD46	AC072051.8	UNK	CCCTATTTTCCCCATCATCA	AAGCAGGCAGATGGTCACTT	FP	55	60	3706	69	166
L1AD47	AC008006.10	18	CGTCACACACATAACCAGAG	GATCAGGAATATGGCAAAGA	FP	55	55	471	212	284
L1AD48	AC027553.6	UNK	TGCATGAAGCACTACTCAAAGA	TGCAAGATGTGTCAGTATTTAGC	FP	60	60	6181	106	226
L1AD49	AC018991.10	UNK	Inserted in repeats		R					
L1AD50	AC008948.8	5	Inserted in repeats		R					
L1AD51	AC008728.7	5	Inserted in repeats		R					
L1AD52	AC093566.3	8	Inserted in repeats		R					
L1AD53	AC020783.8	8	Inserted in repeats							
L1AD54	AC068062.5	10	CCTTTGTTTCTTGGGTGTGG	CCCACATCACCAAACCATTT	FP	60	60	357	128	212
L1AD55	AC064875.5	2	GCCACACTCCTTTGTTTGCT	CAAGCACAAAAGCAGGAACA	FP	60	60	724	193	273
L1AD56	AC073275.8	7	Inserted in repeats		R					
L1AD57	AC010747.10	2	CGGAAAATTTGTTACTTGCT	AGGTATGCTGCATTTCTTTC	FP	55	55	3903	97	272
L1AD58	AC012509.13	2	CCCTGGATGCTGAGTTTCTT	TCCATCTGGCATTGACTCAG	FP	60	60	1062	139	213
L1AD59	AC009964.11	2	TGGGACATTGACTCCTACTC	GGCATAGGTTTCTGGAAGTA	NP		60	760	340	282
L1AD60	AC009961.11	2	Inserted in repeats		R					
L1AD61	AC078851.4	2	TTTATGCTGATCACTGTTCTTC	AACTAGTTGCATCGTGATCATA	FP	60	55	2090	80	208
L1AD62	AC016720.9	2	CTTTCGCATCATCGTAAAGT	ATTGCCAACTGGTTACAAAG	FP	55	55	2886	114	261
L1AD63	AC012492.9	2	AAAAACCCTTTAAGCTCAGT	TGGAAGCATACAAAATGAAA	FP	55	55	6402	342	180
L1AD64	AC069285.8	7	GCCACTGCTAATCAATTCAC	CCAAAGCAGACACAATTTCT	PARALOG	55	60	6131	77	172
L1AD65	AC026029.8	4	TTTCCTCAAAGTTGATGCTC	CCTGGAAGGCATAACTGATA	NP		55	6787	271	575
L1AD66	AC025223.6	2	TATCCAAATATCCCTTGCAG	TTGTAGTTTGTGGAACTGGA	PARALOG	55	55	717	201	197
L1AD67	AC095347.6	12	Inserted in repeats		R					
L1AD68	AC069242.13	3	CCTATGGATGAAAAATGGAC	TCTGAAAATGTTGCCATTG	FP	55	55	294	111	176
L1AD69	AC092325.2	16	Inserted in repeats		R					
L1AD70	AC079841.10	3	TCCAAGAGCAGGCAGTATTA	TTCCTGACTACTCCAGTTCAG	FP					
L1AD72	AC092468.9	3	GTGCAGGTGTAAGGAAGAAA	GTCTTCAAACCAGACTGCAT	FP	55	55	546	93	218
L1AD73	AC097657.3	4	TGATTTGCAGTATTTTTCCTT	GCATGACCCAGATTAGAAAA	FP	55	55	1148	126	168
L1AD74	AC097463.2	2	No results		NR					
L1AD75	AC092018.2	1	TTTCTCTCCCTCAAGCCTTTT	CCAAAATTCATGCTGGGAAC	IF	60	60	1636	129	124
L1AD76	AC027345.5	4	AAACCTCCCTTTAGTCTCCA	CACCAGACCCAATTTTTAGA	FP	55	60	4500	221	173
L1AD77	AC097110.1	4	TCAAGGAAGGGAGTTAAAAA	ACTTCTTTCATGCCCCTTAT	HF	55	55	991	729	237
L1AD78	AC026439.4	5	TCTTGAGGCTTGCAAATACT	ATGAGCAACAAGAAATCACC	FP	55	60	1559	295	306
L1AD79	AC016620.7	5	Inserted in repeats		R					
L1AD80	AC092185.3	3	AAGCAGTATGTCTGGCACA	ACAAACTGACACTCCAAACC	FP	60	55	6148	72	197
L1AD82	AC022165.8	16	GGTGTCTCCACAGTTGATTC	CCACCGCCAGATTTTACTA	HF	55	55	2876	117	196
L1AD84	AC090525.8	12	TTCCCTGGGTCACTTTTCTC	TGCCAAATTGCTTTGCATAC	FP	55	55	2068	255	333
L1AD85	AC026120.33	12	Inserted in repeats		R					
L1AD86	AC093865.2	2	ACATGATGTCCCATCTTCCA	AAGAGCCATATGAGAGCTTCC	FP	60	60	1046	271	304
L1AD87	AC022446.6	5	AATTTTTCCCCACATGTTC	ACAGAATGGATTTAGCTTGC	FP	60	60	3761	118	248
L1AD88	AC090519.3	15	Inserted in repeats		R					
L1AD89	AC084819.17	12	Inserted in repeats		R					

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD90	AC092601.3	2	Inserted in repeats		R					
L1AD91	AC008571.6	5	TGCTAAACAGAAGGCACATA	ATAGATCCATCTGCCAAATC	FP	55	60	6266	170	314
L1AD92	AC092638.2	2	TTATCCAAAGAAGGGGAAAGG	TTTGCCTTATAAGCATTGTGAAAA	FP	55	55	6224	181	195
L1AD93	AC096653.1	4	CAACACTCATTACAACCTGTG	CAGAGTTTATCAGCCAGACC	FP	60	60	2336	382	399
L1AD94	AC092581.2	4	CTCCACGTTAACAGATAGGG	TGAGCTTCACTTAACCACTG	FP	60	60	507	341	239
L1AD95	AC096569.1	2	CCAGCACTGATTTCATAGATGC	TTCAGACAACTGAAGTGCCTTT	FP	55	55	6161	89	224
L1AD96	AC092631.1	4	TAATTAGGTAACGCCTGTGG	CAGGAAGCCTAAACTGCTT	IF	60	60	932	98	245
L1AD97	AC008709.6	5	CCCCAGGCTTTTGAAAATTA	ATTCTCGGGGTCCCAATTAC	FP	60	55	6164	111	214
L1AD98	AC060796.7	17	ATGGAAAGGGGAAGATTTTA	GGCTATACTACAACATCCCTCA	FP	55	55	6164	126	203
L1AD99	AC090791.6	11	GTGACACAAAAAGCACAATTAC	CAATGATTCATGAGTTGGAA	FP	55	55	2737	292	303
L1AD100	AC026729.5	5	CCTGGGTCACAATATGAAGA	TCTGATAACCAGAAGATGAAGA	HF	55	60	6324	258	352
L1AD101	AC025467.5	5	AGTCTCCCTTTCAGAAGCA	AATGCTGGGAATCTTACCTC	IF	55	55	6091	66	163
L1AD102	AC025467.5	5	GAATGGGGTGTGCTGTAA	TTTTAACAAGATCCCAGACC	IF	60	55	3721	78	164
L1AD105	AC010275.6	5	ATTCTCGGGGTCCCAATTAC	CCCCAGGCTTTTGAAAATTA	FP	55	55	6164	111	214
L1AD108	AC008550.5	5	CACAATCATACCTTCCCAACTG	CAGATGAGACTTTGGACGTGA	FP	60	60	6154	84	187
L1AD110	AC092721.2	16	ATTTTGTGGTTCAGCATTTT	CATAGAAAAGGGAACAAATGA	FP	60	60	1590	82	226
L1AD111	AC092357.2	16	AAAAGTTGTTTTCCTGATTTTT	AGTTTTCTCTGCAGCTCATC	FP	56	55	6252	188	184
L1AD112	AC034219.5	5	TTTCCAAAAACAGCTAGGAG	CGTTTTTCTAGCTTAGCAATG	FP	55	55	406	106	209
L1AD113	AC005406.2	UNK	ACCTTGATTGCAAATTGTTT	GGTTTCTTGGCCTCTTTACT	FP	60	60	2881	80	189
L1AD114	AC020651.19	3	Inserted in repeats		R					
L1AD115	AC084032.23	12	AACTGCCATGAAAACTTACC	AAAGATTGTCCACATCAAGG	FP	55	60	253	100	190
L1AD116	AC025176.5	5	End of contig		EC					
L1AD117	AC022024.6	10	CAGCAACCATAGGTTGATAAG	GGATTACTGCCCAAAGAAAC	FP	60	60	852	487	310
L1AD118	AC026113.25	12	GACTGCTGGATCAAATGTTAG	ACCACCTTACTCCTGCTACA	R	55	60	6231	188	272
L1AD119	AC024941.30	12	CTTTATTCATGGCAGAAAGC	CTCATGAGATCTGGTTGTTT	R	55	60	1347	112	249
L1AD120	AC066613.7	UNK	Inserted in repeats		R					
L1AD121	AC010857.8	4	Inserted in repeats		R					
L1AD122	AC011712.6	18	CCCAGGGGAATATATGGAAATTA	AATTGAATGCAGATGGTTTTACC	FP	60	55	6631	139	608
L1AD123	AC010928.7	18	CCAGGAGTCAGAGGATTACA	TCTGTTGTGAGAAGCAAATG	FP	60	60	410	98	172
L1AD124	AC013759.6	18	Inserted in repeats		R					
L1AD125	AC013759.6	18	AAACGGTGAAGGAAATGTTG	GACATGAGCAACCATCAGGA	IF	60	60	513	236	309
L1AD126	AC021082.4	5	Inserted in repeats		R					
L1AD127	AC012323.7	16	Inserted in repeats		R					
L1AD128	AC025097.41	UNK	Inserted in repeats		R					
L1AD130	AC039057.8	UNK	Inserted in repeats		R					
L1AD131	AC073258.9	7	Inserted in repeats		R					
L1AD132	AC017014.4	2	GGGAAGTGAAGGCTAACATA	ACCATGGAGCTCAATTTACA	FP	60	60	469	84	187
L1AD133	AC069294.5	7	GGTTGAGAACCACTGTCATAA	GCCAGTGCTTAGATTTACCA	FP	60	60	6212	145	259
L1AD134	AC084732.1	4	CTACCCAGAACAAATGAACAC	AACCTATACGTAGAAAATTGCTG	FP	60	60	1368	475	422
L1AD135	AC008276.4	2	CTCAAGGGTTCTCATCACTAA	GGAAAGGATACCACAATCAA	HF	60	60	1871	87	191
L1AD136	AC017015.4	3	TGGCTGACAAATTGGTGATT	CCCATGTGAACTGCATTGAA	FP	60	60	712	293	217
L1AD137	AC010970.3	Y	Inserted in repeats		R					
L1AD138	AC012284.5	15	GAGCTGAAGAAACAAAGGAA	ACCTCAAATTCATTTTGGAA	FP	55	60	780	75	200

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD139	AC009479.4	Y	Inserted in repeats		R					
L1AD140	AC010722.2	Y	TTCAGGAACATTGCTATGAGGAT	TAGGCATTTATCATGTGCTC	FP	55	55	1643	218	283
L1AD143	AC079175.24	X	CAGTAAACTGGGCTGCTATC	GAGAGTCAAGCAGTGGGTAA	FP	55	60	5078	80	208
L1AD144	AC023842.5	8	CACAAGATTCAATACCTGAGTGACA	TGGGCATTACTAGTTGAACCTAAAG	FP			1641	141	261
L1AD145	AC087883.12	12	GAAGGAAGCCCCCATATGAT	GAGGTGAAAGGCCATTAAAGAA	FP	60	55	473	147	243
L1AD146	AF280107.1	UNK	End of contig		EC					
L1AD147	AC063951.22	12	End of contig		EC					
L1AD148	AC024060.5	3	AACTTCCTTAGGACCTCATTT	TGTGTTTAACGTTCTAAACCTG	FP	60	60	1361	65	229
L1AD149	AC087433.4	15	CCGAAACACAGATAAGCACT	AGTGTAAAAATCTGCATAGCC	FP	55	55	2160	508	274
L1AD150	AC073572.19	12	ATTCCCCCAATTCTCCAAAA	GCAAGGGCCAACTATGCTAA	FP	55	55	1195	124	187
L1AD151	AC023795.18	12	Inserted in repeats		R					
L1AD152	AC079865.14	12	GGGAGATCCAGACATACAAC	TGTGTAACTCTTTTGCGATG	FP	60	60	569	369	341
L1AD153	AC058784.17	13	Inserted in repeats		R					
L1AD154	AC023812.7	3	ACCTCTACCTTACCACACCA	CCTAACTCAGGTCATTCTGC	FP	60	60	1475	175	260
L1AD155	AC018923.21	3	Inserted in repeats		R					
L1AD156	AC008436.5	5	Inserted in repeats		R					
L1AD159	AC008496.5	5	Inserted in repeats		R					
L1AD160	AC034194.4	3	AGAGCTACATGGCTAAATGC	TCTGCAGTTTTAACACCTCTT	IF	60	55	543	238	261
L1AD161	AC011546.6	19	Inserted in repeats		R					
L1AD162	AC020717.3	X	TTCCTATAGGCTTGAATGGA	TTTTGGTGCCCAATAGTATC	FP	55	60	2923	198	219
L1AD163	AC007132.3	2	CCCAGTATGTCCCTCACTCAG	TAGGCAAACCCCAATTGAAA	FP			6359	315	351
L1AD164	AC006968.2	X	TTCCCTGTCCAATGTAAAGAA	AAAGTGCATATTGCACAGGA	FP	55	55	836	107	158
L1AD165	AC010685.3	Y	Inserted in repeats		R					
L1AD166	AC010889.3	Y	CCCTAACATTTCAAAATGCACTG	ATTTTTCCAACTACTGGCACTCA	FP	60	60	1256	162	214
L1AD167	AC006334.3	7	Inserted in repeats		R					
L1AD168	AC009489.3	Y	TGCCTTTATAATATGGAAATGCAG	TGCTCATGGAGTCAGAATATGAA	FP	55	55	1080	196	183
L1AD169	AC011745.4	Y	TCCCATTGCATTTAGCAGATT	AGGCCTGTATTTCAATTGTGCTT	FP	60	55	3676	95	265
L1AD170	AC007278.3	2	GTCTATTAATCCCCCTCCAC	CAACGTTGAAAAGATGTAGAGA	FP	60	52	6149	87	174
L1AD171	AC006992.2	7	TGGAACTATTTCAGGAAATTAAA	AACAAGGGGGAAGAGAATAA	FP	55	55	6278	197	234
L1AD172	AC006362.2	7	Inserted in repeats		R					
L1AD173	AC015542.17	3	TTCCAATATACTTTGCCCTTA	AGTAGGCATCAGCAACAGTC	FP	55	55	546	393	322
L1AD174	AC022013.3	3	TTTGGGGAGAACTATCTGTG	GCTTGGACATTGGAATTTT	FP	60	54	399	118	188
L1AD176	AC026204.4	3	GCACTCTCATTTACTGCTGA	CCACCTTTTACTATTTTGGTG	IF	60	55	838	494	195
L1AD177	AC018514.7	14	ACCAGATGGAAGCTAGATGA	AAGTTTCCAAGGGAAATCAG	FP	60	55	6370	256	373
L1AD178	AC058791.3	7	ATTGTTTAGGGGAAAAGGAC	CCAAAAGCAGGTTAATTCTC	FP	55	55	629	203	322
L1AD179	AC013738.4	10	ACTCCACTTTAATTCGCAAG	GAAGGCGAGAAACTGTAGAA	FP	55	60	1056	113	289
L1AD180	AL627250.8	X	Inserted in repeats		R					
L1AD181	AL449304.19	9	TTCCATAGCCATTGATTACA	AATTTTCAGGCACGTTTTTA	FP	55	55	652	286	446
L1AD182	AL137787.11	X	Inserted in repeats		R					
L1AD183	AL445312.5	X	GTCCAGAAGTCTCTCCTGTT	CGATTGCAGGCTTTCTAATA	FP	55	60	2873	105	413
L1AD184	AL360020.15	9	Inserted in repeats		R					
L1AD185	AL391260.13	10	TTCTGTAGGGCTCCTGACTA	ATTCACAGTTCCCCGTAGTA	FP	60	55	7905	185	1829
L1AD186	AC016951.9	3	ACTTGAAATTGGGGTAGATG	ATTTTCTAGAGGGCTCCTTG	IF	60	59	843	190	206

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD187	AL365258.24	1	Inserted in repeats		R					
L1AD188	AL603765.6	1	Inserted in repeats		R					
L1AD189	AL596326.5	1	TGTTTCATGGAGTGTATTTCA	TGCAATGTTAGAAGAAGTGG	HF	55	55	456	198	289
L1AD190	AL606752.11	1	GCTTGACACATAGTGCTTGA	AAATGTGGCATTATTTTCACT	FP	60	60	462	250	193
L1AD191	AL589877.13	X	ACCCAGAAACGCATATACAC	GCAAATTGCAACAAGATAAA	FP	55	55	1926	591	352
L1AD192	AL513493.11	1	TGTCCAATTAAAAGGCACAT	TGGAATATCTTTTTCTGCCTA	FP	55	60	941	134	322
L1AD193	AL359733.15	1	TCTTTTACTCCCAAAAGGAA	TTGGGTAGATGAAGATGACC	NP		55	1900	260	292
L1AD194	AL357873.17	1	GCCCTGGATGTAGTGTATGT	CTCTCTCTTCATCCGTTCAG	FP	55	55	974	144	256
L1AD195	AL592494.4	1	No results		NR	55	55			
L1AD196	Z82209.2	X	TTCTCTCCTAACCCTCTTGG	TTTAGGGTATGCGGTAGAAG	FP	60	55	6581	349	385
L1AD197	AL354949.10	1	GAAACTGAGATTCACGGAAG	AGTTTCTCATCCCACCTTCT	FP	60	60	6437	360	467
L1AD198	AL138785.8	1	GCTTCACCTCACTAGCCTTA	CTCACAAAGCAGCATTTACA	FP	60	60	456	87	163
L1AD199	AL445197.4	1	TTCAGCATATCTGCAAAGTG	GAAAGGATTCTCATTTCCTG	FP	55	60	626	216	341
L1AD200	AL136224.24	6	CAGTCTATCAATTCCTGTTGG	TGATCATCCAGCTCAATTACT	FP	60	55	2353	472	440
L1AD201	AL607144.5	13	CAGACTTGGGCATCTTTTAG	AAAACATCAGGGCCAAATA	FP	55	57	1328	148	178
L1AD202	AL513324.8	10	Inserted in repeats		R					
L1AD203	AL390834.24	10	Inserted in repeats		R					
L1AD204	AF245226.1	21	Inserted in repeats		R					
L1AD205	AL596342.3	1	GACTCTTCCCCTTGAGAATC	GCATGCCTACGATCTCTTAT	FP	55	55	381	222	253
L1AD206	AL603902.4	6	Inserted in repeats		R					
L1AD207	AL592067.4	13	ATTTAGGTATGCGTTTCAGC	ACATCTCTTCATGCCTTCAG	FP	55	55	999	422	238
L1AD208	AL353743.22	9	ATCTCCTATCCCCTTAGCTG	AACCCAAGAGTCACAGTTGA	HF	60	60	1978	530	280
L1AD209	AL139282.10	1	TTGAGTCAAGGAAAAATAATGA	AAAGCAAGGCAGGTATGTTA	FP	60	60	1667	214	245
L1AD210	AL512504.9	X	Inserted in repeats		R					
L1AD211	AL590439.12	10	ATATTGATTTGGCATCCTGA	GTAAACGTTCTAGCCAAAGC	FP	60	60	6207	155	169
L1AD212	AC007347.3	16	CACGGGAGAAGATTTATGTC	TTGTACCTACTCCACCCAAG	FP	54	55	6400	210	310
L1AD213	AC007262.4	14	GCCATAAACAGAAAACCATT	GTTGCAGAAATAACAGCACA	IF	60	60	494	182	294
L1AD214	AC007221.2	16	GCAGTCAACATCTTCCAGTA	TGAGCTAGAATCCCAAAGAT	FP	55	60	6267	135	324
L1AD215	AC007115.1	12	TGAAGAACCTTCACGTAAGAA	AAATATGATGCTTTGCTTCC	FP	60	55	556	176	362
L1AD216	AC006143.1	X	GAGGCTTACTGGAAGCATAG	CTCACGGTTGATGTCACTTT	FP	60	60	1494	430	520
L1AD217	AC011594.8	12	CTGGCCAAAGAGGTAGTTT	CAAAAGAGCATGGTACTGGT	NP		55	7620	479	537
L1AD218	AC004141.1	7	TCCTTAACCTAGTTGCTCCA	AGGGTACATTGAAGTTGAGG	NR	60	60	624	340	458
L1AD219	AC002076.1	7	AGGGAATATTTGGGACATCT	CCCCACCACACTAGAAACTA	IF	60	60	6418	354	391
L1AD220	AC003085.1	7	CCAGGGAACTTGATTTTAGA	CAATTGGATAAGAGGGACTG	FP	60	55	6500	303	199
L1AD221	AC004161.1	UNK	Inserted in repeats		R					
L1AD222	AC006204.1	7	TTTGGAAGCTTCACTTTAGC	TGGCCTTAATATTTTAGCAAC	FP	60	60	590	167	246
L1AD224	AL356096.11	13	Inserted in repeats		R					
L1AD225	AL513355.16	10	CGGTTCTAAACACCATTTGT	TTATGGCCCTTAATTTCATC	FP	60	60	1739	177	192
L1AD226	AL358873.25	6	GCATCTTTGAATCAACAAGTC	TGTATCTAACTATTCCCAGTGATT	FP	60	55	986	238	751
L1AD227	AC004822.1	X	TTGAGAGCATCCATATTTCC	CCAACCTCAGATTACCAAGA	FP	55	60	768	115	202
L1AD228	AC005053.1	7	Inserted in repeats		R					
L1AD229	AL450312.10	9	Inserted in repeats		R					
L1AD230*	AL583806.7	6	GCAATCCATAGACAACCAAT	AGGAGGAATATGCAAACTGA	HF	55	55	2249	599	338

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD232	AL583825.8	1	TCCCAGAACTACCTCATAACA	GAGGAAGACAGTGTCACAGA	IF	60	60	1162	219	329
L1AD233	AF207955.1	21	AGGGGTAGATTTTGTTCAGA	AGGACCATTTGCAATGTTAG	FP	60	60	1283	747	667
L1AD234	AL391992.8	10	TGGCTAGTCACCCTAAAAGA	GTTTTATAGGCTTGCATTGG	FP	60	55	6487	388	360
L1AD235	AL160234.3	14	GGAGCTATTAAGCCACAAAA	GAGAGGGTATCCTCGTCTTA	FP	55	60	6771	694	326
L1AD236	AL079307.7	14	GAATGGGGAATTATACGTGA	GTAAGGCACTTGGAAATGTG	FP	60	60	6260	196	295
L1AD237	AL162431.17	1	AAGTGAATGTGGATTTACCC	TCTCAAGGAAATCAGCTCTT	FP	60	60	6499	435	324
L1AD238	AL389895.3	14	ACTTTTATGCCTGAAACCTG	ATCCTTTCTCAGAGGGATCT	FP	60	60	6370	325	278
L1AD239	AL357045.10	1	Inserted in repeats		R					
L1AD240	AL591770.1	14	GTCTCAGACACACAAGCTCA	TTGGCCACTCATCTATCTTT	HF	60	60	540	222	258
L1AD241	AL512310.3	14	Inserted in repeats		R					
L1AD242	AL136960.4	13	CCCCTGAAGAGTCCATATAA	CCTAACAGTCAGGAAAGCTG	FP	55	55	6347	288	197
L1AD243	AL445466.9	1	CTGCTTGTCTTTGGTCTGAT	GTGATCCTGTAGGCCTTCTT	FP	60	60	2933	410	1229
L1AD244	AL512790.1	14	GCATCCGTTTCTCTGATG	TGCAGATTGTACAGAAAAGC	FP	60	60	1394	166	296
L1AD245	AL136295.3	14	ACTTTAGGATTCCGTGGTTT	AATGCTGTTAGAGGAGGATTC	FP	55	60	2193	482	222
L1AD246	AL391838.9	13	Inserted in repeats		R					
L1AD247	AL512662.8	UNK	Inserted in repeats		R					
L1AD248	AL138694.18	UNK	Inserted in repeats		R					
L1AD249	AL133241.3	14	Inserted in repeats		R					
L1AD250	AL121852.3	14	CCCTCAAGAACGATTTTATG	TGTCTAGAATGTTCCCCTTT	FP	60	60	6397	280	237
L1AD251	AL117191.6	14	CTGTGGAGGAAACATTGAAG	TCACACTCAAAGACTCCTTTC	IF	60	60	1995	172	288
L1AD252	AL590370.2	6	GTGAAGGGCACTGGTTATTA	TAATGAAATCGGACCTGTCT	FP	60	60	6498	408	202
L1AD253	AL163613.2	14	TTGCCTAGCTTTTCTACCAG	TTCAAGCTACCTTCTCAAGC	IF	60	60	1369	726	180
L1AD254	AL118557.5	14	ACCTTGACATTCCTGCAA	AATCCACCTGCAGACATTAC	FP	60	60	1000	143	514
L1AD255	AL117693.5	14	TCATTGTTCTATCCATGCCTTTT	GTAGGTTTGGGGCTGGAAAT	IF	55	60	961	197	228
L1AD256	AL161804.4	14	Inserted in repeats		R					
L1AD257	AL359545.12	10	Inserted in repeats		R					
L1AD258	AL358293.4	14	GGTTCAATTGAGCGTTACTT	TGCTGATATAGCACCTAGCA	FP	60	60	6800	735	300
L1AD259	AL158111.5	14	Inserted in repeats		R					
L1AD260	AL133238.3	14	GGTGGATGTATCCATTGTTT	TTTATGCATGCAAGAAATGA	FP	55	55	627	436	464
L1AD261	AL049838.3	14	CTATGGACCCATCTGACTGT	AGTTATTAAACCGGCCACTA	FP	60	60	6269	222	245
L1AD262	AC006568.7	4	ACACGGAGACACTTCAAATC	ACCCGTTATTGTGTTCAGAC	FP	60	60	6424	363	407
L1AD263	AL355481.12	13	GGCTACTTTGGCTTCTGTAA	ATTTGCTCAAACATTTCTGG	FP	55	55	5616	511	531
L1AD264	AL031681.16	20	GGGGAAGTTCCTCCTATATT	AAATGGTAGGTTGGTTTATCA	IF	60	60	1699	501	350
L1AD265	AL589693.3	6	ATAAATTTTCAGGCCTTTCC	GAACAAATTAGACACCATAAGGA	FP	60	60	6218	172	189
L1AD266	AL365508.19	6	Inserted in repeats		R					
L1AD267	AL445258.4	X	Inserted in repeats		R					
L1AD268	AL034425.9	20	GTTTAACCCAGCTGTCCAT	TCCTGTCTCATTTGCTTACC	FP	60	60	2022	361	395
L1AD269	AL136090.12	20	TGACATGGGAGCAATAATAGT	CAGGTGAAATGTATTGAAGGA	FP	55	55	1933	315	371
L1AD270	AL135936.11	20	Inserted in repeats		R					
L1AD271	AL390057.12	6	Inserted in repeats		NR					
L1AD272	AL161901.18	13	Inserted in repeats		R					
L1AD273	AC006947.2	17	GCCTGCTACATGTTCAGAT	CCATCCTTTCTGGAGTGAT	FP	60	60	6252	214	243
L1AD274	AL161938.6	20	Inserted in repeats		R					

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD275	AL157380.15	X	Inserted in repeats		R					
L1AD276	AL031679.1	20	ATTCTTCCTGCCACCTTATG	TTAATAGCTGAGCATCATGG	FP	60	60	993	492	372
L1AD277	AC006265.1	17	GTACAAACCATGGACCAGTT	ATGCAAGTATTTGGCATCTT	FP	55	60	6451	386	239
L1AD278	AL121757.7	UNK	Inserted in repeats		R					
L1AD279	AL157881.14	UNK	Inserted in repeats		R					
L1AD280	AC006131.1	UNK	Inserted in repeats		R					
L1AD281	AF036938.1	X	CAGAGTGAAGTGCTTGGTTT	CTTAATATTTGGGCCATGC	NR	60	55	1342	494	590
L1AD282	AL450303.10	1	No results		NR					
L1AD283	AL358434.6	UNK	Inserted in repeats		R					
L1AD284	AL357141.8	6	No results		NR					
L1AD285	AL359252.17	6	ATCCAATCACCATCATCAGT	ACCTGTGTTCCTATCTTTGC	FP	55	55	823	423	272
L1AD286	AL354937.12	9	TTTAACAACGCACACTTAGC	ATTAAGCAATGGCAGGAAT	FP	60	60	1385	337	444
L1AD287	AL356430.19	13	TTGAAATCAATAATGAGGGATA	AACATCAGTCAGCTAAAGCA	FP	55	55	518	277	256
L1AD288	AL121574.19	UNK	Inserted in repeats		R					
L1AD289	AL390039.10	UNK	Inserted in repeats		R					
L1AD290	AL158167.15	10	CCATGCCTCAACATCTCA	ACCTTCCTTATCTTCCCTTG	IF	60	60	750	175	237
L1AD291	AL157398.6	10	TGGAAAAATATCCCATAATGA	TTTCAGATGGTTTTTCAACA	FP	55	55	6277	180	311
L1AD292	AL136970.8	6	GGCAAATTGAGTCAAAGATG	AACTCATTCACAGTAGCAACAA	FP	60	60	6281	206	200
L1AD293	AL136117.12	6	TGGGAATCAGGAAATTTAAC	CCTATTTCTTGGGTTTTCTG	FP	60	60	2300	199	429
L1AD294	AL356286.8	X	Inserted in repeats		R					
L1AD295	AL158201.19	X	AAAGAAAGAAAACACCCACA	CTCACGTATTATTCCGATTTG	NP		60	2579	245	699
L1AD296	AL136441.16	13	AACCAAGGACTTACACATGC	ACTACCACTCATCCAGCAAA	FP	60	60	6518	461	261
L1AD297	AL357499.10	UNK	Inserted in repeats		R					
L1AD298	AL136455.6	1	TGCCACATCTGTTCAGTAAA	GAAATAGGCTCGTTTTCTCT	FP	60	60	1906	399	351
L1AD299	AL359502.14	13	TTAATGCAAGCAGAGTTTCC	TAAGAACCCATGGTCCAGTA	FP	60	55	6269	180	291
L1AD301	AL139334.10	6	AGTTGTCTGAGGAAACACCA	TACGCAGCATCAAGTAAAGA	FP	60	60	1823	700	288
L1AD303	AL139092.12	6	Inserted in repeats		R					
L1AD304	AC005358.1	17	ATCAGTGGTTCTTTGTCCTG	AGCAGTTCACAGTCCTTAGC	FP	55	55	1230	226	248
L1AD305	AC004768.1	5	GCCAGGAGATAATTTGTAGC	TACCTTGCCAGTAACCTTCT	FP	60	60	2726	386	330
L1AD306	AC004389.1	X	End of contig		EC					
L1AD307	AC004074.1	X	Inserted in repeats		R					
L1AD308	AC004523.1	UNK	Inserted in repeats		R					
L1AD309	AL138702.8	13	GCATTGCAGAAGAAAGCTA	TACCTCCAAGGCAAAACTTA	FP	60	60	1547	273	293
L1AD310	AL121946.20	6	CAACACACGTACAGGTATGC	TTAGCCTCTGTCTTTTGTGC	IF	60	55	6557	519	372
L1AD311	AL135932.7	11	TGACCTGTTCTGATGATTGA	CTTCTCAGGGTATCTGTCCA	FP	55	55	2281	271	327
L1AD312	AL136086.8	1	TTGGGGATAACTTTAACTGC	CCTTTTCATCCTCATGTTTT	IF	55	60	6284	228	209
L1AD313	AL137026.21	10	GCAGGAGAGAGTAAAGGGTTA	TGACAACCACTGCTATCAAG	FP	60	60	1382	86	165
L1AD314	AL121938.10	6	GGCTCAGGGAGATTTGATA	TCTGTTGTACTCTTTCAGGAACT	FP	60	55	3462	311	322
L1AD315	AL121933.15	6	GGTAACTAAAGCCATTGCAG	TATCTTTGGATGCTGCATAA	FP	55	55	2636	429	316
L1AD316	AL133547.16	9	Inserted in repeats		R					
L1AD317	AL157378.8	6	Inserted in repeats		R					
L1AD318	AL355871.5	1	TGTGGCTAATTCTGAGACCT	ACATGAGTTATCGTGGCATC	IF	60	60	631	176	175
L1AD319	AL157361.6	13	CCCAATGAACCTGTTGTAGT	GGATTTACATGCCACTTAGG	FP	55	60	392	188	241

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD320	AL157360.8	UNK	TCCAATGTTCTCTTAGAGGAGT	TCAACATGCAAAAGACTGAA	FP	60	55	489	114	248
L1AD321	AL139115.5	9	CTTGTCCATTTTCTCCACTG	CAACCCAGTAACTCCACTTC	FP	60	60	1193	80	200
L1AD322	AL049796.28	1	TTCTTCCTGGAAAATTGCTA	TTCCTATGAATCCAGTAGTGC	FP	55	60	6512	434	251
L1AD323	AL117345.21	6	GATGGCTTCAAATCCTTCTT	CACTTCAGATAGAACAAGAGCA	FP	60	55	3744	395	379
L1AD324	AL109920.15	6	TATCATTCCTTCAGGCCATA	GGTGAATGCTTTGGACTTTA	FP	55	60	1568	249	280
L1AD325	Z98950.1	X	TCGGCAGCACATATACTAAA	TCCATAGCCAAGTGAGTTTT	FP	60	55	1001	207	283
L1AD326	AL050309.4	X	Inserted in repeats		R					
L1AD327	AL030998.1	X	AAAACATATTTGGAGGAGCA	GTGACCTGGTGTTTTTGTCT	FP	55	55	6315	202	314
L1AD328	AL133353.6	10	TGCTAATAAAAGCACTCTGAAA	AAGATGGTGAATGTTGTAGGA	FP	55	60	2610	155	284
L1AD329	AL136169.6	UNK	Inserted in repeats		R					
L1AD330	AL133404.8	6	Inserted in repeats		R					
L1AD331	AL136363.4	X	ATTTCTTCTGCAGCTCTGAC	CATGATAACTTTGGTTTGTCAC	FP	60	60	6213	188	279
L1AD332	AL133247.1	2	TGACTGACCACTGTATGGAA	GTGGCTGTTTGGATTCTTTA	FP	60	60	1399	204	247
L1AD333	AL078604.10	6	Inserted in repeats		R					
L1AD334	AL021877.1	22	TTGACTTGTTTAGAAAGGGATT	GGATAAAGCTGAAAGCTCAA	FP	55	60	6322	233	215
L1AD335	Z70758.1	X	TCATCCAGCATTGAATCAG	TTGGTAGAAAGTGAAGTGGAG	FP	60	60	571	199	238
L1AD336	AL096706.10	UNK	Inserted in repeats		R					
L1AD337	AL049589.15	X	Inserted in repeats		R					
L1AD338	AL021069.1	1	AAGAATCCAATTTGCAACAG	TTTGATTCGGATTACACTGA	FP	60	60	6248	173	233
L1AD339	Z97181.1	X	GTTAAAATGCCAGGCTGAT	TGAGAAATGTGTTCTCCAAA	FP	55	55	1169	136	349
L1AD340	AL031117.1	X	Inserted in repeats		R					
L1AD341	AL034348.5	6	TGACTTCCATTTCAGGTACTC	CCACATTAGAGGTTTTCCAA	FP	55	60	4229	143	293
L1AD342	AL022399.2	1	TATGCATTTCCATGACTTGA	GTGGTAGGAGTAGGGGAAAG	FP	60	60	6795	342	708
L1AD343	AL033530.1	1	Inserted in repeats		R					
L1AD344	AL031313.1	X	Inserted in repeats		R					
L1AD345	AL023806.1	6	AGTACCAATGAAGTGCCATT	CAGGAGCATAAATAGGACCA	FP	60	60	1770	379	500
L1AD346	Z80232.1	X	CGGAAAATCCTCAGTCATC	ATGCCACAGCTTAAAAGTTC	FP	60	60	1065	261	309
L1AD347	Z84720.1	X	Inserted in repeats		R					
L1AD348	Z93018.1	X	No results		NR					
L1AD349	Z99128.1	6	AGCACTCCTTTTATGAAGTCAACC	AGAGGAGAGAGTGGTTGATATTGG	FP	55	55	2851	1223	565
L1AD350	Z82170.1	UNK	GGCAGACCAAATGGATTAT	GATCCAAATATCAGACAAAATGT	FP	55	60	6342	288	184
L1AD351	Z95126.1	X	TGACATGCTTCCCTAAGTTT	TATAGAAAGTGAGGCCCAGA	FP	60	60	537	363	313
L1AD352	Z95325.2	X	CTTGCTGAATTAATCCCTTT	GGAAGAAATGATCCATAAGAAA	FP	55	55	3497	355	346
L1AD353	AL022308.1	X	CAAGGGGAAATCTCACAATA	GGACTTTGGGACTTACATCA	PARALOG	55	60	6238	174	263
L1AD354	AL023095.1	X	TCATCTTGCTCCCAAATATC	TCCTTAACACAGTCAAGTGAAC	FP	60	60	4839	170	338
L1AD355	Z98948.1	X	No results		NR					
L1AD356	AC000111.1	7	TGTGGCTATGTGAGATGAGA	CCTTAATTTGAGGGGTTTTT	FP	55	55	4633	326	385
L1AD357	AP004241.2	11	CATAGGACGTTCAAGTGTGA	ATTGTCTATGGCTGCTTTCT	FP	60	55	765	387	593
L1AD358	AP002803.3	11	AGGTTTTGAGGTTTGCTGTA	TCCCAATAATCACTTTCCAC	FP	55	55	6274	205	264
L1AD359	AP002002.4	11	AAGGGCATATAAAACTGGTG	GCACCCATTAACTCATCATT	FP	55	60	6460	356	328
L1AD360	AP000764.4	11	CCATGCTTTCCACTCTTTAT	GCAGAAAAGGGTGTTCATA	FP	60	60	379	179	240
L1AD361	AP002784.3	11	GGAAAAATGACAGTCAGGAG	GCCTACCCAATGAATATCCT	HF	60	60	1031	149	258
L1AD362	AP003719.3	11	Inserted in repeats		R					

(*continued*)

Table 3 Continued

Name	Accession	Chrm. loc.[b]	Forward primer	Reverse primer	Human diversity[c]	AT (F,R)[d]	AT (ACG)[d]	PCR product sizes[a]		
								Filled	Empty	Subfamily specific
L1AD363	AP000811.4	11	CCATTACTTGAAGCAGAACC	CTGTGGGTCTCAGATCATTT	FP	55	55	6419	367	175
L1AD364	AP001977.4	11	TAAACTGGGGCTAGAAGTCA	CCAATTGAGAACCATCTTGT	FP	55	55	6335	383	344
L1AD365	AP002982.2	8	ACAGAGATTTCCTGGGCACT	TCAAACTGCATGCAAAATCC	FP	55	55	811	109	208
L1AD367	AP000789.4	11	CCAACAGGGATCAAAGGTTC	GCCACCTTGAGTTGGTGAAG	FP	55	55	378	147	175
L1AD368	AP002006.5	11	TTTCTTTTCCTACTCTCCCTCTC	GAGAAATAAAGGCAATTGCTCAC	NP		55	4593	186	922
L1AD369	AP001485.4	11	AAAACATATAAGCGGCCAAC	CAGCACCTGTTATGGTTTGA	FP	60	55	2437	466	187
L1AD370	AP000462.2	11	TAAGAAGAGGGGAGGAGACT	GCCTCTATGAAGCAGGTATG	FP	55	60	793	178	237
L1AD371	AP001709.1	21	CTAAATTGCTCCATTCCTTG	ATCACTGTAGGGTGATCCAG	HF	55	55	2525	581	562
L1AD372	AP001678.1	21	CTTACGCCTCAATTATCTGG	TGCAATTGATCTTACAAGGA	FP	55	55	2325	280	269
L1AD373	AP001674.1	21	CAAATAGCCAGCACAATATG	TTGTCATTGGTCTTTTGTCA	FP	55	60	823	165	226
L1AD374	AP001669.1	21	Inserted in repeats		R					
L1AD375	AB009801.1	14	AATCCACCTGCAGACATTAC	AGAACATCCCCTATCCAAAC	FP	55	55	688	87	202
L1AD382	Z95325.2	X	Inserted in repeats		R					
L1AD383	AC090791.6	11	TGGTGGTCTCAGAGTAAACA	ACCCAAAACATCATTAGTGC	FP	60	60	1642	117	1026
L1AD384	AL136441.16	13	Inserted in repeats		R					
L1AD385	AP003123.2	11	GCACAGGTTTATCTCCTTGA	ATTGAAGACCTGCAATTTGT	FP	55	55	6379	284	287
L1AD386	AC114975.2	5	Inserted in repeats		R					
L1ADY8	AC010970.3	Y	TCACACGTATCCCTTTGCAG	TTTTCTGTGAAACATCTTGGAGA	FP	55	55	1813	115	204

* Indicates L1 preTa element identified by Ovchinnikov 2002 (Ref. 28).

[a] PCR product sizes: empty product size is calculated computationally by removing the L1 preTa elements and one direct repeat from identified filled site. Subfamily-specific product size is calculated from internal subfamily-specific primer located in the 3′ UTR to the proximal 3′ primer. In cases where target site duplication sequences were not found flanking the element PCR product sizes may vary from those reported.

[b] Chromosomal location was determined from accession information or by PCR analysis of NIGMS monochromosomal hybrid cell line DNA samples. L1 elements with unknown locations are denoted UNK.

[c] Elements at the end of sequencing contigs are denoted (EC), those residing in other repeats (R), those having paralogs (PARALOG), and elements with inconclusive PCR results (NR). Elements represented here are classified according to allele frequency as: high frequency (HF), intermediate (IF), no pre-integration site in primate samples tested (NP), or as fixed present (FP) insertions. Fixed present: every individual tested had the LINE element in both chromosomes. Intermediate frequency insertion polymorphism: the element is present in more than 30% of alleles tested and no more than 70% of the alleles. High frequency insertion polymorphism: the element is present in more than 70% but not all alleles tested. Indeterminable data is denoted (−).

[d] Amplification of each locus required 2:30 minutes at 94 °C initial denaturing, and 32 cycles for one minute at 94 °C, one minute at annealing temperature (AT), and one minute elongation at 72 °C. A final extension time of ten minutes at 72 °C was also used.

Table 4. Autosomal preTa L1 allele frequency and heterozyosity

	African American genotypes					Asian genotypes					European genotypes					South American genotypes					
Element	+/+	+/−	−/−	f[a]	Het[b]	+/+	+/−	−/−	f[a]	Het[b]	+/+	+/−	−/−	f[a]	Het[b]	+/+	+/−	−/−	f[a]	Het[b]	Avg Het[c]
L1AD10	0	5	14	0.13	0.23	0	8	12	0.20	0.33	3	7	7	0.38	0.49	3	7	10	0.33	0.45	0.37
L1AD14	9	10	1	0.70	0.43	4	8	7	0.42	0.50	16	4	0	0.90	0.18	17	2	1	0.90	0.18	0.33
L1AD19	13	7	0	0.83	0.30	15	2	0	0.94	0.11	14	6	0	0.85	0.26	14	6	0	0.85	0.26	0.23
L1AD20	18	2	0	0.95	0.10	19	1	0	0.98	0.05	16	0	0	1.00	0.00	19	0	0	1.00	0.00	0.04
L1AD75	0	5	15	0.13	0.22	0	1	18	0.03	0.05	1	9	9	0.29	0.42	0	9	11	0.23	0.36	0.26
L1AD77	1	5	11	0.21	0.34	0	1	19	0.03	0.05	0	3	13	0.09	0.18	0	6	12	0.17	0.29	0.21
L1AD82	19	1	0	0.98	0.05	17	0	0	1.00	0.00	20	0	0	1.00	0.00	19	1	0	0.98	0.05	0.03
L1AD96	13	5	2	0.78	0.36	15	1	0	0.97	0.06	5	10	5	0.50	0.51	11	7	2	0.73	0.41	0.34
L1AD100	19	0	0	1.00	0.00	19	0	1	0.95	0.10	20	0	0	1.00	0.00	20	0	0	1.00	0.00	0.02
L1AD101	16	4	0	0.90	0.18	10	5	0	0.83	0.29	13	6	1	0.80	0.33	11	9	2	0.70	0.43	0.31
L1AD102	14	0	0	1.00	0.00	14	1	0	0.97	0.07	12	1	2	0.83	0.29	0	4	16	0.10	0.18	0.13
L1AD125	12	7	1	0.78	0.36	14	6	0	0.85	0.26	20	0	0	1.00	0.00	19	1	0	0.98	0.05	0.17
L1AD135	19	1	0	0.98	0.05	20	0	0	1.00	0.00	20	0	0	1.00	0.00	20	0	0	1.00	0.00	0.01
L1AD160	11	5	4	0.68	0.45	5	11	4	0.53	0.51	4	12	1	0.59	0.50	4	8	3	0.53	0.51	0.49
L1AD176	7	3	2	0.71	0.43	2	9	5	0.41	0.50	0	1	15	0.03	0.06	1	0	11	0.08	0.16	0.29
L1AD186	4	7	8	0.39	0.49	14	5	1	0.83	0.30	5	10	2	0.59	0.50	4	11	5	0.48	0.51	0.45
L1AD189	14	5	0	0.87	0.23	19	0	0	1.00	0.00	20	0	0	1.00	0.00	19	1	0	0.98	0.05	0.07
L1AD208	14	6	0	0.85	0.26	19	0	0	1.00	0.00	14	0	0	1.00	0.00	14	0	0	1.00	0.00	0.07
L1AD213	7	9	3	0.61	0.49	2	12	5	0.42	0.50	2	2	5	0.33	0.47	8	5	7	0.53	0.51	0.49
L1AD219	3	14	3	0.50	0.51	0	10	10	0.25	0.38	1	5	14	0.18	0.30	2	11	7	0.38	0.48	0.42
L1AD230	14	6	0	0.85	0.26	19	0	0	1.00	0.00	20	0	0	1.00	0.00	20	0	0	1.00	0.00	0.07
L1AD232	13	7	0	0.83	0.30	8	7	3	0.64	0.47	12	2	0	0.93	0.14	13	4	1	0.83	0.29	0.30
L1AD240	13	3	0	0.91	0.18	20	0	0	1.00	0.00	13	0	0	1.00	0.00	20	0	0	1.00	0.00	0.04
L1AD251	3	9	7	0.39	0.49	10	8	2	0.70	0.43	14	4	0	0.89	0.20	8	11	1	0.68	0.45	0.39
L1AD253	11	6	3	0.70	0.43	0	14	5	0.37	0.48	4	8	7	0.42	0.50	0	6	14	0.15	0.26	0.42
L1AD255	1	8	10	0.26	0.40	1	9	10	0.28	0.41	6	7	7	0.48	0.51	3	14	3	0.50	0.51	0.46
L1AD264	4	10	6	0.45	0.51	2	9	8	0.34	0.46	2	7	7	0.34	0.47	3	11	6	0.43	0.50	0.48
L1AD290	7	12	1	0.65	0.47	4	8	7	0.42	0.50	3	13	0	0.59	0.50	6	9	5	0.53	0.51	0.49
L1AD310	5	6	7	0.44	0.51	0	5	15	0.13	0.22	5	2	5	0.50	0.52	6	5	7	0.47	0.51	0.44
L1AD312	0	4	16	0.10	0.18	11	6	2	0.74	0.40	2	9	5	0.41	0.50	2	7	9	0.31	0.44	0.38
L1AD318	4	12	4	0.50	0.51	2	12	6	0.40	0.49	4	8	8	0.40	0.49	3	11	6	0.43	0.50	0.50
L1AD361	17	3	0	0.93	0.14	19	0	0	1.00	0.00	20	0	0	1.00	0.00	20	0	0	1.00	0.00	0.04
L1AD371	15	5	0	0.88	0.22	18	2	0	0.95	0.10	20	0	0	1.00	0.00	20	0	0	1.00	0.00	0.08

[a] f represents the frequency of the element.
[b] This is unbiased heterozygosity.
[c] Average heterozygosity is the average heterozygosity for all populations.

along with genomic sequence at its 3 prime end. This sequence then integrates at a different genomic location, resulting in duplication of the source L1 sequence and the 3 prime genomic sequence flanked by target site duplications.[16–18] We have identified 50 3 prime transduction events mediated by preTa L1 elements and believe that these elements have transduced approximately 10,400 total bases of sequence with one transduction event responsible for duplicating a region over 1600 bp. The diversity observed in the tails of the L1 elements is not surprising, since previous studies have shown an association as well as direct evidence that simple sequence repeat motifs present in the 3 prime tail of mobile elements can mutate, serving as nuclei for the generation of simple sequence repeats.[41–43] A complete list of the preTa elements involved in transduction events is located at our web site†.

L1 associated human genomic diversity

Of the 362 preTa L1 elements isolated *in silico*, 102 of the elements were inserted into other repetitive regions of the genome such that flanking unique sequence PCR primers could not be designed. Six additional elements resided at the end of sequencing contigs in GenBank and lacked unique flanking sequence information, making PCR primer design in this region impossible. The remaining 254 were analyzed using a subfamily-specific PCR assay and flanking unique sequence primers as previously described[28] (summarized in Table 2). Three elements out of 254, produced inconclusive PCR results because of the amplification of paralogous genomic sequences as described previously.[44] Nine elements produced non-specific PCR results, and were excluded from further analysis. Another nine elements produced subfamily-specific PCR products in all human samples tested, but did not produce pre-integration sites in both human and non-human primate genomes. This may be the result of some type of large deletion event that occurred in the human genome and not in the genome of non-human primates, making the non-human primate pre-integration site much larger than expected and not detectable by our assay as reported previously.[19] Alternatively this could also be the result of mutations in the oligonucleotide hybridization sites rendering them ineffective for PCR. In addition, we identified 36 preTa L1 elements that mapped to the X chromosome and eight that mapped to the Y chromosome, all of which were fixed present in the individuals tested (Table 3). The human genomic diversity associated with the autosomal preTa L1 elements is shown in Tables 3 and 4.

A total of 293 (254-9-9-3) preTa L1 elements produced unambiguous results when analyzed by a two-step PCR assay across 80 individuals from four geographically diverse human populations with 33 (14%) being polymorphic with respect to insertion presence/absence (Tables 3 and 4). Examples of human genomic diversity associated with preTa L1 insertion polymorphisms are shown in Figure 4(a) and (b). Of the preTa L1 elements, 11 were high frequency insertion polymorphisms with L1 element allele frequencies greater than 0.70, so that most of the individuals were homozygous (+/+) for the presence of the LINE element. Of the polymorphic elements, 22 were intermediate frequency, with a LINE element allele frequency greater than 0.30 but less than 0.70 across the diverse human populations sampled. None of the L1 preTa elements tested had insertion allele frequencies less than 0.30. One possible explanation for the absence of low frequency preTa insertion polymorphisms would be that the preTa subfamily has largely undergone retrotranspositional quiescence and is no longer generating new copies. As a result, the number of low frequency preTa insertion polymorphisms in the human genome would be limited. It is also possible that the newly integrated preTa L1 elements are removed from the human genome as a result of negative selection. However, we consider the former explanation more likely based upon the threefold higher levels of insertion polymorphism in the Ta subfamily as compared to the preTa subfamily (45% *versus* 15%) as well as the previously reported frequency distribution of Ta L1 insertion polymorphisms in the human genome.[24]

A total of 200 preTa L1 elements were fixed present in the human genome. These elements are likely to be slightly older than their polymorphic counterparts, having inserted into the human genome prior to the radiation of humans from Africa. Overall, the unbiased heterozygosity values across all of the L1 elements subjected to PCR analysis were similar across the four populations, with values of 0.306 in African Americans, 0.243 in Asians, 0.252 in Europeans, and 0.269 in South Americans with the African American population being the most diverse with respect to preTa L1 alleles (Table 4). However, several of the polymorphic elements individually exhibited unbiased heterozygosity values that approached 0.5, the theoretical maximum for bi-allelic loci.

In order to determine whether the LINE insertion polymorphisms were in Hardy–Weinberg Equilibrium (HWE) we compared expected genotype frequencies with observed genotype frequency using chi-square tests for goodness of fit. A total of 132 chi-square tests for goodness of fit are theoretically possible. However, 28 of the comparisons involved populations that were monomorphic for the presence of the L1 insertion leaving 104 possible tests. A total of 23 deviations from Hardy–Weinberg expectations were observed in the comparisons. A total of 18 of the deviations were the result of low expected genotype frequencies. Of the remaining five tests that deviated from HWE, none clustered by population or locus. This deviation is not surprising, since a total of 5.15 deviations from HWE would be expected by

chance alone at the 5% significance level. One shortcoming of this method is its inability to deal with low expected genotype frequencies. To further test these polymorphisms for HWE, we performed an exact test for Hardy–Weinberg proportions using the Markov chain test available in the Arlequin program,[45] which is not hindered by low expected frequencies. The exact test showed that none of the 104 comparisons deviated from HWE proportions at the 1% level. Therefore we conclude that the newly identified L1 insertion polymorphisms do not significantly depart from HWE.

Discussion

Here, we report a comprehensive analysis of the dispersion and insertion polymorphism associated with the preTa L1 subfamily within the human genome. We estimate that there are approximately 900 lineage-specific L1 elements present in the entire human genome. In addition, given the median size for preTa and Ta L1 elements (~1600 bp) and a conservative copy number estimate of 900 elements, we estimate that human lineage-specific L1 retrotransposition has been responsible for increasing the size of the human genome by roughly 1.4 million bases.

The level of sequence diversity, estimated age, and the reduction of human genomic variation associated with this L1 subfamily relative to the Ta L1 subfamily provide strong evidence suggesting that the expansion of preTa L1 elements began prior to the expansion of the Ta L1 subfamily that has been analyzed in detail previously.[24,27] However, the expansion of preTa L1 elements also appears to have occurred over a time frame that predated the radiation of humans from Africa and continued until very recently, in fact it may still be occurring at a very low level within the human lineage. Thus, we conclude that the expansion of preTa and Ta L1 elements occurred in an overlapping time frame in the human lineage. The reason(s) for the relative retrotranspositional quiescence of preTa elements remain unknown. However, they may relate to alterations in the ORF2 protein of the preTa elements, decreased transcription from the preTa "source" elements or a decrease in the ability of the elements to undergo target-primed reverse transcription.[46] Further studies using *in vitro* systems to measure retrotransposition[25] will be required to definitively address this question.

Sequence analysis of the preTa L1 insertions suggests that they have a slight preference for integrating into regions of the genome with low GC content. This observation is contradictory to that previously reported,[43] but is in agreement with results obtained by The International Human Genome Sequencing Consortium.[3] The reason for this integration site preference is unclear, but may result from a subtle sequence preference of the preTa-encoded endonuclease. Alternatively, this observation may reflect limitations on L1 preTa insertion events imposed by chromatin organization. However, it is likely that both factors, as well as others not mentioned here, are important in determining where in the human genome young L1 elements will integrate. It is also interesting to note that some preTa L1 insertions have occurred adjacent to known genes. The persistence of these newly integrated preTa L1 elements in these regions of the human genome is most likely indicative that they have had no negative effects with respect to the function of these genes.

Of the essentially 105 full length L1 preTa elements identified, 29 have both open reading frames intact and are putatively retrotransposition-competent elements. The data collected from the L1 preTa subfamily along with the L1Hs Ta subfamily (44 elements) yield a computational estimate of 73 active L1 elements within the genome that is comparable to previous estimates of the number of potentially active L1 elements in the human genome.[26] Collectively, these data suggest that L1 elements from multiple subfamilies may still be capable of retrotransposition within the human lineage. In addition, it is also important to mention that those full-length elements that no longer have intact open reading frames could have previously served as active source or driver genes for the expansion of pre Ta L1 elements, but have accumulated mutations over time that subsequently inactivated them.

The computational identification approach described here provides an efficient and high-throughput method for recovering preTa L1 elements from the human genome, some of which are polymorphic for insertion presence/absence in individual human genomes. Individual L1 insertion polymorphisms identified, similar to other mobile element insertion polymorphisms, are the products of unique insertion events within the human genome. Because each L1 element integrates only once into the human genome, individuals that share L1 insertions (and insertion polymorphisms) inherited them from a common ancestor, making the L1 filled sites identical by descent.[24,28] This distinguishes L1 insertion polymorphisms from other types of genetic variation that may not be derived from a single ancestral allele, including microsatellites[47] and restriction fragment length polymorphisms.[47,48] In addition, the ancestral state of an L1 insertion is known to be the absence of the L1 element. Therefore the 33 new L1 insertion polymorphisms reported here appear to have genetic properties similar to the previously identified *Alu*[44,49–53] and L1[24,27,28] insertion polymorphisms and provide a unique form of genetic variation present in the human population that will serve as an additional source of identical by descent genomic variability for the study of human population relationships.

Materials and Methods

Cell lines and DNA samples

The cell lines used to isolate primate DNA samples were as follows: human (*Homo sapiens*) HeLa (ATCC CCL2), common chimpanzee (*Pan troglodytes*) Wes (ATCC CRL1609), pygmy chimpanzee (*Pan paniscus*) Coriell Cell Repository Number AG05253, gorilla (*Gorilla gorilla*) Lowland Gorilla (Coriell Cell Repository Number AG05251B), green monkey (*Cercopithecus aethiops*) ATCC CCL70, owl monkey (*Aotus trivirgatus*) OWK (OWKidney) ATCC CRL 1556, and Orangutan (*Pongo pygmaeus*) (Coriell Primate Panel PRP00001 Cell Repository Number NG12256). Cell lines were maintained as directed by the source and DNA isolations were performed using Wizard genomic DNA purification (Promega). Human DNA samples from the European, African American, and Asian population groups were isolated from peripheral blood lymphocytes[54] available from previous studies.[50] South American Human DNA was obtained from Coriell Human Variation Panels HD17 and HD18.

Computational analyses

The draft sequence of the human genome was screened using the Basic Local Alignment Search Tool (BLAST)[30] available at the National Center of Biotechnology Information Genomic Blast page †. A 19 bp oligonucleotide, 5′-CCTAATGCTAGATGACACG-3′ that is diagnostic for the preTa subfamily was used to query the Human Genome database with the following optional parameters: filter none; advanced options −e 0.1, −v 600, −b 600. Copy number estimates were determined from BLAST search results. Sequences containing exact matches were subjected to additional analysis as outlined below.

A sequence region of 9000–10,000 bases, including the match and 1000–2000 bases of flanking unique sequence were annotated using RepeatMasker version 7/16/00 from the University of Washington Genome Center Server‡ or Censor from the Genetic Information Research Institute§.[55] These programs annotate repeat sequence content and were used to confirm the presence of preTa L1 elements and regions of unique sequence flanking the elements. PCR primers flanking each L1 element were designed using Primer3 software available at the Whitehead Institute for Biomedical Research‖ and were complementary to the unique sequence regions flanking each L1 element. The resultant primers were screened with standard nucleotide-nucleotide BLAST [blastn] against the non-redundant (nr) and high-throughput (htgs) sequence databases to ensure they resided in unique DNA sequences. Primers residing in repetitive sequence regions were discarded and new primers designed if possible. A complete list of all the L1 elements identified using this approach is available from our website‡. Individual L1 DNA sequences were aligned using MegAlign with the ClustalW algorithm and the default settings (DNAstar version 5.0 for Windows) followed by manual refinement.

PCR amplification

PCR amplification of 255 individual L1 elements was carried out in 25 μl reactions containing 20–100 ng of template DNA, 40 pM of each oligonucleotide primer (Table 1), 200 μM dNTPs, in 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8.4) and *Taq* DNA polymerase (1.25 units). Each sample was subjected to the following amplification for 32 cycles: an initial denaturation of 150 seconds at 94 °C, one minute denaturation at 94 °C, one minute at the annealing temperature (specific for each locus), and an extension at 72 °C for one minute. Following the cycles a final extension was performed at 72 °C for ten minutes. For analysis, 20 μl of each sample was fractionated on a 2% (w/v) agarose gel with 0.05 μg/ml ethidium bromide. PCR products were directly visualized using UV fluorescence. The human genomic diversity associated with each L1 preTa element was determined by the amplification of 20 individuals from each of four geographically distinct populations (African American, Asian, European, and South American) for a total of 160 chromosomes.

† http://www.ncbi.nlm.nih.gov/BLAST/

‡ http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker

§ http://www.girinst.org/Censor_Server-Data_Entry_Forms.html

‖ http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgiand

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Supplementary Material is available on Science Direct

Table 1 Supplementary Data. L1Hs Ta PCR Primers, Chromosomal Locations, and PCR Product Sizes

Name	Accession	Chr. Loc.[1]	5' Primer Sequence (5'-3')	3' Primer Sequence (5'-3')	A.T.[2]	Human Diversity[3]	Product Sizes[4]		
							Filled	Empty	Subfamily Specific
L1HS1	AC010739	2	AGGGAATGCTTATATTGTTGATGAG	ACTTCCTTCAGGGTTAATAGCAAAG	60	FP	3877	159	224+
L1HS2	AC010305	16	ACCAAATATCTGGACACTTTCTGG	GAAGTCAGCAGTGGTTAATTTTACA	60	IF	6131	74	171
L1HS3	AC008572	5	GCTTCTAGAATTGGAAGTAATATGG	AGTAGCCTTGAATCATCTTTTG	56	FP	656	95	422+
L1HS4	AC009494	Y	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	467	-	-
L1HS5	AC020647	12	TCAACTACAAAGTTGAAGAATAGG	GTTTCCATCAACAAGATCATGTCAAG	58	LF	546	376	455+
L1HS6	AC016138	3	TTTATTTCCCTGCATCTGATTA	CCTGTTATTAGATAATGAGTTCTAGTC	54	HF	402	122	219+
L1HS7	AC004773	7q11	CCTTAGACATATTCTTGGAAATAG	CCAGAATATTTGGGTATTTCATCTG	58	HF	326	169	256+
L1HS8#	AC004491	7q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1689	-	-
L1HS9#	AC004694	7p	TCTTTCAATGGAAACAAGAGGTATC	AGGGAGAGGGACACTGAGTTTAT	59	FP	6126	74	178
L1HS10*	AF149774	7p	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6076	-	-
L1HS11	AL049842	6q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	667	-	-
L1HS12*	AC007538	Xq28	GTTAAAGCAATCAAGCAATCTACTG	TAACAAGGCCACTGTAGAAAAGATT	59	FP	6188	104	209
L1HS13#	AC007938	7q31	ATGGGAAGGAACCCCATCTAT	AATTACTCCTCTCTTTGGCCTGTT	59	HF	745	128	220+
L1HS14	L05367	17q	AAGTGGATTAACAGTAACATACAGA	CCAAGCTGATAACTGATTATCTCA	55	IF	601	251	158
L1HS15#	AC007556	2	AATGCATACCCATGAGGACAA	ATGGTGTTGCACAACAAAAGAA	60	HF	6167	126	197
L1HS16	AP000220	21q	CCCTCACAGAGTGCTTGGTAA	GGGAAGGTAGGAAAACAGATT	56	IF	368	101	207+
L1HS17#	AC007486	X	GCATCCCTAAAGCAATAATCCA	GGAATTTTCCACTTGTGGTGTC	60	Paralog	4286	90	170+
L1HS18	AC005798	4	TTGAACAGCTTAGACTCGTCAGATA	GCAGTTAGACAGGAAAACAGAAAGA	60	HF	6174	87	212
L1HS19	AC007876	Y	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6115	-	-
L1HS20	AC009241	2	AATGGAAGAGCTCTCAAATTCCTTA	GCAACCATTCAAAAATTTACAACAG	61	IF	2302	62	181
L1HS21	AC008277	2	GTGTTGGCATATTTCTATTCG	TAAAGGCTGAACTTTGCATTG	57	LF	2606	84	178+
L1HS22	AC010682	Y	GCTCTCGGGTTCTTCTACCTCT	TCTACTGTTCCATGCAATAGATGTG	60	NR	3216	266	249+
L1HS24#*	AC004554	Xp22	GTGTATTTTGCCTTTTGAACCAA	CAAAAACTTGTTTCACTTGATTTTTAG	59	IF	6148	101	181+
L1HS25#*	AC002385	7q31	GAGGACCTTATTCATTTATTGC	CCATCTGAGCTTTAGTTTTGTCATA	60	FP	6140	94	191+
L1HS26#	AC003689	11q12	GCTTCAAGCTTAAAAGATGTAGACT	CCTACCCAAGTATCCACTGTCC	60	IF	2652	589	420+
L1HS27	AC007736	2	AGAACGTTGCCACATTATTTTGA	GTAGGAAGGTCTGGACTGGAGTATT	58	FP	3667	68	214+
L1HS28*@	AC002980	Xp22	CTTTTGTGACACTGGATTTCTAGC	CACTGTATATTGGAGCTGTTTTTCC	58	IF	6531	282	373
L1HS29#	AC005090	7p	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1476	-	-
L1HS30#	AL022166	Xp11	CCCTAAACAGAAAGGAAAATGAGAC	TCCTCATTGTGGTTCAAGGTTATAC	60	IF	4795	97	175+
L1HS31@	AC019212	X	GACAACACAAAGAAAACCCAAGAT	CTTATGTCCCAAAGCTAGTGAGTGA	56	FP	2317	86	176
L1HS32#	AC004911	7q	TCTCTAATCCAGCCTTTCAATTC	TGTTTCTTTTCCTGTGTGTTTCC	57	IF	463	280	384
L1HS34@	AC002122	5p15	ATGTCTGTCTTGACATTCCTAAGC	AATATGTAGAATGGCACAGGCTTC	58	IF	2177	284	328
L1HS35*	AC010081	Y	CTACCACATAACTGAGTGACAGTTT	CAATGTGCATCCATATAGCTGTGTT	61	FP	6308	233	239
L1HS36#	AC004000	Xq23	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6038	-	-
L1HS37	AC003080	7q31	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6017	-	-
L1HS38#	AC004142	7q31	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-

L1HS39	AC005690	4	AGAACCAATCTTGCCCACAC	TGAGGAGTTTCTGAGTAACCTGGTA	60	HF	6337	155	189
L1HS41	AF222686	Xp11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1959	-	-
L1HS42	AC020925	5	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	580	-	-
L1HS43	AF172277	7q21	TTTATTGCACCTCCTGGTAAAGTAG	AGAGCACCATTAAACAACACAAGAT	58	IF	6157	89	191
L1HS44#	AC004883	7q	TAGCTGTGCTTGTTATGTCCAGTT	GAATGAGTTTTGTGTGGTTCTGTG	57	VLF	2288	478	615+
L1HS45#	AC004865	1	AATAGGCCCAGCTATTAGATTTAGC	CCTTTAAACCTTTGAACACGATTT	53	FP	329	81	150+
L1HS46#*	AC006027	7p	CCTGTGTTCCTTTTGTAATCC	CAAATGTCTCTTCAAGGACTG	55	HF	6382	326	183+
L1HS47	AC006986	Y	AGTCAAATGATTTTTAACTGCTG	GAGGGCAAGATCATGAAACA	58	Paralog	6177	86	230+
L1HS48#	AC005105	7p	CGAAAAGCTTAGGAAACTGTTTGT	TAAGCAATCTTCAGTTTAGGAAA	58	FP	1242	810	420
L1HS49	AC010202	12q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	612	-	-
L1HS50	AF198097	Xp11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6308	-	-
L1HS51	AC008055	12q22	GCCCCTTACGTTAGAATAGAAAC	TGGATTGGTCCATACTACTGT	55	FP	1094	272	239+
L1HS55#*	AC004704	4q25	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6063	-	-
L1HS56#	AC005908	12p13	CCATTCATCAGCCATTTGCTA	GTGGCTTTAAAACAACGAGATG	59	FP	6545	459	494+
L1HS57#	AC006222	4	CAGCAAGACTCTGTCTCTAAAATGAT	GGACTTGAATTTGGTCTTGTTTCTA	59	LF	589	195	284+
L1HS58#	AC005939	17	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6101	-	-
L1HS59	AC003678	11q12	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2081	-	-
L1HS60#	AC006465	7p	GAAGTATGGAAATTGAGTCACA	CCCTAAGCTGTATCACTTTAAAACA	56	FP	445	104	246+
L1HS61#	AC002288	16p12	ACGTTTGTGCTTCACTCTAAGTTCT	CAAAATACCGGGATTATAGTTGTGA	57	FP	353	68	175+
L1HS62	AC006840	4	ATTAAAAGGAATGGACATGCAACAC	AATCTCAAAAGCTTCCTTGCACT	60	FP	6282	182	256+
L1HS63	AC023423	Y	AAGAAAGTGTTGTCAGAGAGTGTGA	AGGCCATTGGTCAGTCATAATTT	60	Paralog	6160	115	200
L1HS65#	AC004053	4q25	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1781	-	-
L1HS68#*	AC004200	6p21	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6242	-	-
L1HS69#@	AC004220	5	GGATGTTGATGATGGAGTCAGTC	TAACCATTTGAAACCATTAGAGGTC	60	FP	1410	76	180
L1HS70#@	AL049588	Xq	GTTCATTTGAGTGAGGGTACTGTCT	TAAGTCCCAAAAATTGCATCC	59	IF	3174	175	256+
L1HS72	AL133413	9q	CTGAGATGAGACAGCAGGTCTTC	TCTGCTGAGATTCTTCCATTTACC	60	FP	825	147	221
L1HS73	AC018822	3p	ATAAGGAGCCTAGGGAAGAACTTTT	CAAGCATGCCTGAAACATCTAT	55	HF	1126	462	162+
L1HS74*	AC011990	17	CTGGACGTATTTCTTACAGAGTTGA	CCCTAAGTTATTTTCCTTGAGGCTA	60	LF	6163	125	186+
L1HS76	U08211	X	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS77#@	AB020867	8p	TTCCTAAATGGCCTTACTATCCTTT	TCAGAAGTGCTAACAACTCTAGTAGGA	58	HF	990	78	233
L1HS78#	AP000084	21q22	TAGTACCTCCCTTAAAGAGCTG	GAGGAAAAGAAAAGTGCCTGATA	59	IF	374	107	175+
L1HS80#	AC017051.4	UL	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1823	-	-
L1HS81	AP000962	21q21	AAGTGTTATATATTGGAGCAATTC	ACAAGACAATGCCAATTTTAAGAGA	60	FP	848	148	401
L1HS83#	AJ001189	Xq12	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS85	AC008132	22q11	TTTGTATGCCTTGTGTTTTGTATTG	AGGAGAGTCTCATCTCCAGAGTTAC	58	LF	593	79	183+
L1HS86*	AL121825	22	GCAGTATCAGGAAATGCAATACAC	GGGATTCAGTCACCTTTATTAGACA	60	HF	6154	410	180+
L1HS87*	AL078622	22	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6065	-	-
L1HS91#	Z84572	13q12	ATACGTGCAAAACAGGAGATTTGA	TGTTTATGGTGAAGGATAAGTCTCA	59	FP	1619	78	167
L1HS92	AL022153	Xq	ACAATCCCTACTTCAGAAAGTT	CAACACTTTGATCATGAATAATAGCTC	57	FP	859	121	206
L1HS93	Z95325	Xq21	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	4882	-	-

L1HS94#*	AL031586	Xq	TCGTATGAATAACCTTGTGTTCTTG	TTTAGATCCTCGTCACTCAAAGTGT	57	FP	6250	151	264
L1HS95#	AL023284	6q	GGAAATTCTCAAGCTCAAGTTAAAA	CTTTTAAAGTGTGTTCTCACAGTGG	60	FP	717	119	320+
L1HS97#	AL030998	Xq	AACCAAACCCACAATCAGTAGAA	CTAGCTAAAGGTTTGCTATTTTT	58	FP	1640	182	407+
L1HS98#	AL022099	6p	ATCTGCATTGGGCCAAGTTTT	TCTCCTGTAAGACAGCACCATA	60	FP	1561	129	242+
L1HS99#	AL022726	6p	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6290	-	-
L1HS100	Z98754	Xq	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6161	-	-
L1HS101#	Z72519	X	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS102#	AL096677	20p	CCATTTGCCATAAATAAAGGCATC	ACTGTTACAAGTTTCCCCAAATGT	59	FP	6741	611	542
L1HS103*	AL121591	20	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6019	-	-
L1HS104#	AL096799	20	GAGATGTGGTTTTGTTTGAACTG	GCAGCTCACATAGTTTAGAGAAGAT	59	IF	6196	131	219+
L1HS106	AL117339	10	CTGACTGTTGAAACTTCTCCATTG	CAATAGACATGAAGGCATGGAAG	57	FP	3103	378	345
L1HS108*	AL031768	6p	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6091	-	-
L1HS109*	AL137191	14	GCCTTTCTATCTTTTGCTCTTGGT	GACACATACCAATTACAGGCAAAG	59	FP	6549	501	381+
L1HS110#*	AL078623	20	GGATTCTGACCTTATTCTAACAGCA	AGTTGACTGTTGGTGTTGATTGTGT	56	HF	6263	212	253
L1HS111#	AC002069	7q21	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	535	-	-
L1HS112	AC018755	19	AGGTTCCATCTCTAATACTGGATAA	TGATCACTTTGTTGTTAAGATGGAG	60	LF	1686	102	170
L1HS113@	AL133386	6p	AGTTTTGGCCTGAGAGAGAAGTAGA	GGTAGGCTAGAGATCCCTTCAATTA	55	FP	405	184	328+
L1HS115	AL132639	14	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	182	-	-
L1HS116	AC024610	18	CTGTGCACTTTTCCATATGTTTGAC	TCTAATCTATGGTGGATGCTCTTTC	56	FP	252	76	189
L1HS117#*	AC005885	12q	TGCAGTGTTCTATTTATGTCGTAGGT	CGAGAGAGGGAGGAAAGTGAG	57	IF	6629	535	176+
L1HS118	AC020599	4	ATGCCAGAAATACCTCTTTTACCTT	CTAAGTGCAATTCTCTCAGATTTTG	60	IF	6321	286	277
L1HS119#	AC005739	5	GGCTTATTTAGAGCACCTGGATTTA	GAGATCCAAAGCTTATGCTGTAAGT	60	FP	904	243	257+
L1HS123#	AC005350	5q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	397	-	-
L1HS124#	AC004499	20q	TGACATAATTAATGGAGAAAACCAG	GAGATCCCTGTCCTTGTGTGAT	60	FP	749	515	373+
L1HS125	AF001905	Xq25	CCTCACGTTTCTCCACATTGTA	TTCTGGCCTTCATAGTGTTTTA	60	HF	332	96	169
L1HS126#	AC004784	19q13	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1552	-	-
L1HS127	AC004384	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	225	-	-
L1HS129#	AC003100	4q25	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1132	-	-
L1HS130	AL133320	1p	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6066	-	-
L1HS131	AL163152	14	TTGACTGTGTACTGCCAGTCTCT	GTAACCTACCAGTTTACAGTTACC	58	IF	381	179	212
L1HS132	AP001693	21	CCCTGATACACCAGTATATCTTA	GAAAAGAAAAGTGCCTGATA	56	IF	753	486	173+
L1HS133	AC008716	5	CATGGTGTCCCAGTGTTAAAAA	TATCTCTTACCTCTTCTTGCCCATA	59	FP	3351	821	738+
L1HS134	AF265340	16	CACAGTCAACTCAACCACTGAATAA	AAGGAGATGGAAGTAAGTGCAAAC	60	FP	751	433	603+
L1HS135	AL137804	11p	TTTTTGAAGGGAGTACAGTAATAGGT	GCCTTCCATAGTTCCTATTTGC	58	FP	6475	429	500+
L1HS136	AL157791	14	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	175	-	-
L1HS137	AL157879	5	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6057	-	-
L1HS150	AP000966	21q21	CAAGAACAACTGAAAAATGCAGAT	CCCCTCAGTCTCTGGTTACCTA	58	FP	642	89	141+
L1HS151	AC019205	6	CTTTGATCAGTTCTTGGAACTAGGA	CCTCTATGCCTTATTCATGCTTATC	60	FP	573	405	476+
L1HS153	Z84814	6p	CCAATTCACTTTGTCTCCTAGAAAT	AGTTCACGAAGTTGAAAGCTTATGT	60	IF	931	169	219
L1HS155	AC019050	2	TGGCATGTCAATATATACCTGAAGA	GGAAAACAGAAATAAAAGACGGACA	60	FP	7004	596	720

L1HS157#	ALO49842	6q	ATTCAAGTTCCAGTAAGCTGTGTTT	GAACTTTGGAAAATTCACAACTACC	60	HF	892	143	245
L1HS158#	AC008467	5	CAGCCCAGAGTAGTTCATGTTTT	GAAGGAAAAGGAGCTGCTTAGATA	59	IF	6194	147	207+
L1HS159	AC009976	Y	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1439	-	-
L1HS160	AL121938	6q	CTAAATAGGCAGAGGAAAGGAAAAC	TAAACTTCCAAGAGATCAGCACTTC	60	HF	1071	99	225+
L1HS162	AC009404	2	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	463	-	-
L1HS163	AL139114	9p	GGGACAGGGGTTAAGATTTTATTTT	AGTTCTCAACTGTAAAGGCAGTGTC	60	IF	2898	85	251
L1HS164	AB045357	1q	GGAAGGAAGTGGGGATAATAAGTAA	CCCAATTCAGTTTCTTCATTCTATG	60	FP	1507	193	267+
L1HS165	AC011666	1q21	CACAGTGATGGAGTTACAATCTTTG	GCTTTAAAGTCAGACAGGCTTGAGT	62	FP	1509	200	276+
L1HS166*	AC021017	8	TGCCTGAAATGCTATTGGTAGTATC	GTGCCCAGCCCATAATATAAA	60	IF	6204	102	251
L1HS167	AC018637	7	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2975	-	-
L1HS168	AC009492	2	CTTTTTCAAGGCCATCTGTGAG	AATCCTTACAATGAAAAGGGTGT	61	FP	666	97	180
L1HS169	AL118519	6q	TATTGAGGTGTAACCAGCATACAAT	CCACACGAAAGATATATGAATTGC	60	IF	6289	214	288
L1HS171	AL137145	10	GAAAGTTCATGAAAGTTGTGATGC	ACAAGAGAATCTATCTCCTGAAGAA	60	IF	6157	91	198
L1HS172	AL133479	9p	CTAAGATCAGTCACAGGCTTAATGA	CAGGTGCAAGTGGTTTAATTTTC	60	IF	1326	111	193+
L1HS173	AL359218	14	CACCATCTAGTGATTTTATGTTCTGC	AATAATCCCCATTGACTGTGTACTG	55	HF	319	123	217+
L1HS174	AJ271735	Xq	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	3252	-	-
L1HS175	AL136382	1p	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	717	-	-
L1HS176	AC025819	Y	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1522	-	-
L1HS177	AC017015	18	CAAGTTCCTCACCAAATGAAACTAC	TCCATTTTACTGATGTTGAATAGGC	58	HF	693	165	273+
L1HS178	AC023480	3p	GAATATTGAGCTTTCTTCACCTTT	CAAGCATGCCTGAAACATCTAT	60	HF	508	54	162+
L1HS179	AC017089	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	3573	-	-
L1HS180	AC009276	7	GGAGTGTAGAATACTGGGGAAAATC	CTTATTTCCCAATGAGCCCTGTA	56	IF	507	84	225+
L1HS181	AC025759	5	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1179	-	-
L1HS183#	AC000100	19	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS184	AL450108	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6094	-	-
L1HS185	AL157837	1q	CTGGCAGTTCCCTCAATGTAA	GAGTAGCTAGCAAAACAGGTAATGAA	60	FP	604	108	214+
L1HS186	AL359332	14	GGTCTAACAATATTCATGATGC	CCTCTTTTACCCTGTGAAGAAAAT	60	FP	6313	249	205+
L1HS187	AL357153	14	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6059	-	-
L1HS189	AL512407	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	907	-	-
L1HS190	AC073893	Y	TCTACTGTTCCATGCAATAGATGTG	GGGTTCTTCTACCTCTGCATAACT	57	NR	3243	190	331
L1HS191	AC007972	Y	TCCTCCAAGACCCTCTAAAATAAAT	TTTTGTCTTCCCTGAGTAAATTCTG	60	FP	2645	122	251
L1HS192*	AC018680	4	TTTCACTTTTTCTATGGTGATGAGG	CTTAGAATGTTACACTTTTCCGACA	60	FP	6218	155	196
L1HS193	AC018503	3	CTACAGTGGCATTTCTTTAGGACAA	TATACAACAGAACTGAATCACTGAC	60	FP	6296	239	288
L1HS195	AC044791	15	GCTTACATCTCAAATTCTGGTACCTT	TGTAAGAGCCAAAGCCTTTTAAACT	60	FP	1521	150	209
L1HS196	AC025263	12	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6071	-	-
L1HS197	AC027332	5	TGGAGTAGAATTCAAGCAAACTGAA	AGAGTTTATGATAGGTCCCCATTCT	60	HF	6226	97	260+
L1HS200	AC009892	19	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1686	-	-
L1HS202	AL391097	20	TTGTACCTATGATTTGTGTGATAGGC	GCTCTACATAAAAAGATGTTCACCA	60	FP	990	754	435
L1HS203	AL354750	10	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	152	-	-
L1HS204	AL157815	13q	ACTAGTTGATGACAAACTGGATGTG	GAGTGGCATAATCAATTGCTAGAGA	60	FP	647	126	182+

L1HS206	AL355382	6	GTTTGTCAAGTGACAGGAATCTCTT	GCTAAGTCATCAATAAGCCCCTAAT	60	FP	2704	154	186
L1HS207*	AL354861	9	CTTTGCATATCTCTGTCATCCTACA	GATGAGATCATTCACACACTTTCTG	60	FP	6208	164	170
L1HS208	AL354793	X	AACATTGGGAGAAGTTTGCAGTAT	CCAAGTTGTTAAGCACTCCATAGTT	60	FP	6639	570	689+
L1HS209	AL158159	9	GATGAGTTATCTTTGACGCTTTGAC	TGATAGATGAATGAGCTTTATGGTC	57	FP	508	118	213+
L1HS210	AL135908	6	ATGTGGGGAAGATGAAGAAATC	GAAAACCCCACTATAGGAGTAAATTG	59	NR	5322	132	564
L1HS211	AC079598	12	TCTATCGTCTCTGTCTTCTTAATGC	AATGACACTCTGCCTTCAGACTTAG	57	NR	3001	275	407+
L1HS212	AL157700	Xq	TTCTAGCCCTCTACTAATGTCCTTG	TTCTAAGGTAGCTGCAGATAAGTGG	60	FP	1045	184	234+
L1HS213	AC087432	3p	AATGCCTGATAAAAGTAGACACACC	GTGGGAATATATCTTCTTGGGTTT	60	HF	1710	89	188+
L1HS214	AC007483	3	TAGCTGAGAAACCATAAGCCTAGAA	ACCTGAATGTCCACTCATTCACT	60	HF	4159	328	330+
L1HS215	AC037423	9	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1162	-	-
L1HS216	AC023880	7	CTATACCAAATGCAGTCAGGATGTT	TCCCATAACTCTGTCACACTAGAAA	59	FP	714	197	228
L1HS217	AC073148	7	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6063	-	-
L1HS218	AC016910	2	TCTTACAGCACTATTCAGTGTTTGC	TTCCTCTCAAGGAACTCAAACC	60	FP	6136	82	174
L1HS219	AC021020	3	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6096	-	-
L1HS220*	AC016635	5	ATTGGCCTTCAGAAGTGATTAAGAC	TAGATAGCCAGACAAACAAACCTTG	60	LF	6244	135	260+
L1HS222	AL445932	6	TCTTTCTCCTCTTGTAATGTCTCAG	AAGATACTGTGCTTCACTCTTCTGG	60	LF	6195	118	238
L1HS223	AL450488	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	4210	-	-
L1HS224	AL358934	9	GATCTGAATCTTTGCTCTCCAGATA	ACGTGGTACAAAAGAAAACACTGTC	60	FP	1121	126	215
L1HS225	AL445523	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	3537	-	-
L1HS226@	AL353153	6	CCCTAAGCCTGTCAGAAGTTAGTATC	GCCATGAAAGATAAGGAGATAAGAG	60	LF	2114	120	359
L1HS227	AL157701	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	518	-	-
L1HS228	AL353657	13q	AATATCCACTACCCAATTCCATAGG	GCTGCAATTTAGCAGGATTTCT	60	HF	1383	184	205
L1HS230	AL359174	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1291	-	-
L1HS231	AL354896	13	GAGTATGAGAGCTCTGCTTTCTGTC	CTTGAAGGACTGGGATACTTGAAA	60	HF	2289	379	481
L1HS232	AL365367	1p32	TGTCACTCCAGTGATAGAAGCTAGA	ACAGTTAACTTCAAGGCAGGTTGAC	60	FP	1181	69	214+
L1HS233	AL357507	6	TAGTTGTCTACAACCAAGTGCTGAG	TCTGCATAGATCAGGAATTCTAAGG	59	IF	1232	81	174
L1HS234*	AL356438	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6092	-	-
L1HS235*	AL158193	13	ACAGGATCTTAAGGTTGAAGGTTTG	GGTTCTACCCAAAGTAGTCAAGAAA	59	IF	6441	420	179
L1HS236	AL365400	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1711	-	-
L1HS238	AL357519	6	GCAGGTAGGATACATGTAAGCATTT	ATCACAGCAATGGCATATCATC	60	FP	2155	374	360+
L1HS240*	AL137845	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6103	-	-
L1HS241	AP003112	8q23	GATAATCAGGTGATTGTGAACTGTG	CTACCACCCTTTTTACTCCCTTTAC	60	FP	366	148	206+
L1HS242#	Z80899	6p21	AGTTCACGGTCTCTATCTCTCCTTT	AACCTGTCTTTGACTGTTGAGC	58	IF	576	150	277+
L1HS243	AC019041	2	CACTAACATTCTGCATCTCACAATC	GTGGGAGGACATGAATAACACAT	58	FP	6148	96	202
L1HS244	AC009269	15	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	5512	-	-
L1HS245	AC017040	2	AAGGCTCTTTATCACAGGAAGTACC	ACGTTAATCACCGATCATTGC	60	FP	2141	294	263+
L1HS246*	AC068723	15q21	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6224	-	-
L1HS247	AC009274	7	GTGTGAAGTATTACCTCGGTGTTG	CTGTGTGGAGCAATAGTAACCAGAT	60	FP	2238	286	275
L1HS248*	AL360236	6	AGAACAAGTGAGTGGCTAAAACCTC	AGCCAACAATTTTCCCATCTC	60	FP	6705	658	710
L1HS249	AL355852	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1297	-	-

L1HS250	AL162373	13	AGTACCTGGTGAGTTCTCCTCAAC	GGTCTTTTGTGAGATGTCATACCTG	57	FP	2055	110	194+
L1HS251	AL445429	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	757	-	-
L1HS252*	AP002768	11q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6026	-	-
L1HS253	AP001955	4q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1780	-	-
L1HS254	AC013546	8	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	5961	-	-
L1HS255	AC022731	8	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1104	-	-
L1HS256	AC019218	8	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS257	AC016756	8	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS258	AC024905	3	GATTGGACTCCATTTCCTCTTGTAT	ATAAATTCTGGGACCTCTGCTTAAT	57	FP	1717	1011	643
L1HS259	AC020707	9	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1893	-	-
L1HS260	AL354982	9	GGCAACGGAATAATAGCTTCA	GTCAGCACTCCCATCTTAAATGTCT	57	HF	6461	358	510+
L1HS261	AL161631	9	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1904	-	-
L1HS262	AC013579	1	GATCCCTGTGTCTGGAGCACT	GGAATTCATGGAGAAGGTGAGTT	60	FP	1148	97	186
L1HS263	AL356139	9q	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	889	-	-
L1HS264	AL391643	9	GAGGAGGAAGAAGGCTGATAATATG	GACAGCCACTAAGTTAATGAGATCC	60	FP	284	133	174+
L1HS265*	AC018938	9	GCATTATTTCTGGAGCACTCACT	GTCTTGTGCTATTAAGCCTGGTCT	60	FP	6087	105	207
L1HS266	AL137021	9q31	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	207	-	-
L1HS268	AC025428	10	CTTTGCTCTCTTGCTCCATGTAT	TATCTGTTTACCAACCCATCTCACC	60	FP	6235	90	283+
L1HS269	AC020642	10	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS270	AC026989	14	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	313	-	-
L1HS271	AC020644	10	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS272	AL157787	10	CTATGTCCTAGCCTTCCCAGATG	AGAAAAGACAAGACAGGATAGGG	58	FP	1125	201	223+
L1HS273	AL354951	10	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS274	AC027118	10	GCACATGGCTTCTTAGCTAACTT	CTTTCTTGCATAAATGACTCTGTCC	57	FP	2081	611	317
L1HS275	AL590378	10	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1414	-	-
L1HS277	AC026393	10	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	312	-	-
L1HS278*	AC027591	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6020	-	-
L1HS280	AC078971	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6063	-	-
L1HS281	AC037434	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	343	-	-
L1HS282	AP001002	11q	CTTACCTCCAGAGCATGCACATTAT	CCCCTCCTTCTCAATTTAAGGTTAC	61	FP	6448	156	249+
L1HS283	AP000409	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2294	-	-
L1HS284	AC018619	11	AGATAGGAGAATCCTCTGGTCTTCT	CTATTGTTGGGTACTTGGGTCACT	58	FP	1877	174	268+
L1HS285	AC015772	11	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS286	AC011829	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1189	-	-
L1HS287	AC021304	11	CCTTTTATCTGAAATAAGTGGTTGG	CTTCCTTTAGCTGGGCTGTTCTAAG	61	VLF	1693	95	216+
L1HS288*	AC016775	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6081	-	-
L1HS289	AC021245	11	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS290	AP001179	11q	CCTGTCAGTCTTATCTTTGCTCTACA	GGCATAGAGACAAATCCAAATTAAG	60	NR	6537	285	235
L1HS291*	AC025410	6	CTCCCACTACTTTATGGGAAGGT	AGGACTTCCAATTCCTAGTATGCAG	58	HF	5658	216	271+
L1HS292	AC073915	12q	GACTCCACACTAGCTTCTTTGACTT	GAGACTCAGTTGACAAGGAGTTACC	60	FP	1117	117	213

L1HS293	AC026831	12	TTACAATGGATACGTTAGACAGCTC	CCATAATTGGTTAGGATGATGAGAC	60	LF	2517	417	317+
L1HS294	AC027442	12	CTTTACCTGTTCCACTAATCAC	GGCACAAGATGGATATAAAGGA	57	FP	6154	103	168
L1HS295	AC012144	13	GAGGAATGGTTGAACAGCTTG	ATGTGGCTGGAGAAATACCTCTAAG	61	FP	713	100	208+
L1HS297@	AC064857	12	GTCCAGAGTGATGCATTTTATTTGG	GCATAGTCATTTAATGCATGTCAGC	58	FP	771	461	549+
L1HS298	AC025880	12	ATATACCATACTCCTTTCCCCTTCC	TGAGCCCTGTATTTTAATCACTTGT	60	LF	1037	80	235+
L1HS299	AC027287	12	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS300	AC026577	1	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	3364	-	-
L1HS301	AC027382	1	CTATCCCATAGATGGTGGGTAGAAT	GAGGAAATAGCACAGGTATGGTAAA	61	IF	1770	411	431
L1HS302	AL365220	1p21	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2391	-	-
L1HS303	AL451063	1	CTATGTTCTGGGAGAAGAGCTGAT	CTAGGGTCAGAAAGAACTTTGATGT	62	FP	780	87	170
L1HS304	AL354885	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS305	AC016371	1	CAAAAAGCAGCCCTATATTAGC	GCCTGCCTCATTATCTTTCATT	58	FP	3998	415	409+
L1HS306	AL136459	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS307	AL390860	1	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6066	-	-
L1HS308	AL390200	1	CCTACTAGGCCCTCTTCTTTTGTAT	GTCTTGTTGTGCCAGACACTTTA	62	IF	3441	455	652+
L1HS309	AL391904	1	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2161	-	-
L1HS310	AL157946	1p31	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	286	-	-
L1HS311	AL162402	1p13	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	693	-	-
L1HS312	AL139225	1p13	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	783	-	-
L1HS313	AC034157	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS314	AL357975	1	TGGCTAGCAAAAAGGTGGAC	AGGGCAGAGAAAAATGGTCA	58	IF	6215	109	255+
L1HS315	AL139137	1	AAGTCCCAATTCCCTAGTCTGTCT	GACACAGAATCATGTCACAATACCC	61	FP	6286	77	332
L1HS316	AC026905	1	CTTTAGCAGTTTTCATGCCTCCT	AGGTTGATGGTAACCTGTAGGAAC	59	FP	6240	173	245
L1HS317	AL356323	1	CTCTGCCTCAAGTGTGTCTTGACTA	GAGAACACACCCTTGCTCAGTAAAT	59	FP	901	711	626+
L1HS318	AL365225	1	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	5243	-	-
L1HS320	AL357973	1	GGGATTCAAATGGGAAACAAG	CTCCTTTCCAGTATCTGCTCTTATG	60	IF	1748	140	305
L1HS321	AL356455	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS323	AC068071	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS324	AL139284	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS325	AL360154	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS326*	AC025702	1	CTCACCGTTATCAAAGGGTAGAAAC	CTAGCCCCAAATTTGAGAAACAG	60	FP	6250	156	289+
L1HS327	AC018874	1	GGTACAATGTAATCATGGGTTGG	GAGTTAACCGTTAGTCCACAAGATG	58	FP	4695	172	413
L1HS328	AL135842	1q21	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2188	-	-
L1HS329	AC058795	1	CTTCACCTCTGAATGACACACAT	GGCTTCATAATGCATCGCTAA	60	FP	1188	454	365+
L1HS330	AL139285	1p31	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS331	AL138777	1q31	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1064	-	-
L1HS332	AC008110	1	CATGTTAGAACTGGCTCAAGTATCC	CCTGCAGAAATTTGCCTTTAG	58	IF	2850	87	227+
L1HS333	AC023026	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS334	AC026253	2	ACACTTCTGAGAATTTCCCTGTG	TTACTCCCTCTTTACTGTCTTGGTG	60	FP	1095	199	341
L1HS335	AC023434	1	CATGCATCTCTGAACTACTGACTTG	ATAAAAACCTGTTTAGGCCAAGG	60	IF	1276	395	284+

L1HS336	AC013264	1	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS337	AC010890	2	GGTACAATATGAGGCATCACGTA	GTAGCATCCTTTATAGCTTTGCTGA	60	HF	3174	210	329+
L1HS338	AC068953	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS339	AC017035	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS341	AC069384	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS342	AC018591	2	GAGACTCAGTTGACAAGGAGTTACC	AAACAGGACCTGCTGTCCATAA	60	FP	1087	78	183+
L1HS343	AC068572	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS344	AC048375	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS345	AC073509	2	CACAGCATTTACCAAAGCACTC	CTCAGTTCATTGCACAGTTTGG	60	LF	2587	192	229+
L1HS346	AC016674	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS348	AC018378.3	2	GAAATGGGAAGAGGAGTTGACA	CCTATTTTTATCTCAGCTGATGTCG	60	HF	748	283	526+
L1HS349	AC009963	2	GGAGCTGGGAGAATTATTGAAAC	CCACTCTCAACTACTGTCCAACAAG	60	HF	229	114	182
L1HS350	AC022605	2	TGGTATATAGTTCTAAGGACCCACAG	GCTACTTTTGCTTCTGGGTGTT	58	FP	725	243	331+
L1HS351	AC013262	2	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS352	AC073874	2	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	970	-	-
L1HS353	AC019324	2	TCCATGATAGAACACACTCTTCC	AATCCCTGTCAAAACCAATCC	59	HF	1822	426	167
L1HS354	AC012442	2	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6217	-	-
L1HS355	AC011901	2	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6067	-	-
L1HS356	AC009290	2	CATCCTGTTGAAGAACAGAGAGATG	ATAGAGTGACCAGAAACTCCAGAGA	60	FP	6290	156	250+
L1HS358	AC019130	2	GAGACTCTTTGGACTCAGAGTATAACC	AGTCCTGTCATACCAGTTATTGGAC	59	FP	6621	128	673
L1HS359	AC024062	2	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	4808	-	-
L1HS360	AC023416	2	GAGGTCTTTGTGCAGAGGTATAAGA	CTCACCAACATCAGTTTCCTTTG	60	IF	3222	153	218+
L1HS361	AC073642	2	AGCCCATTAGATATATGTGGCTGT	CTTTTTATATTGGTCACCCCCAAC	61	FP	6319	281	372+
L1HS363@	AC010913	2	GTTAGACAGCGACATGCACAG	ACCTCTGTGCCTTACCAAAAAC	60	FP	577	106	198+
L1HS364	AC026860	3	CTTAGCCTCTGTCTTTAGGGAAAAC	CATGACCAACGGTGCATAATA	60	HF	6139	97	170+
L1HS365	AC068355	3	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	888	-	-
L1HS366	AC083853	3	AGAAAACTTCCAGACACCTATCC	CTATGTCCTAGCCTTCCCAGATG	60	FP	1088	163	183
L1HS367	AC078805	3	GACTCATATTACCCTGGACAACAAC	AGTCTCTCCTTGCTCAGTTTGGTAG	60	FP	6784	83	401+
L1HS368	AC023144	3	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	168	-	-
L1HS369	AC076971	3q	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS370	AC068365	3	GCAATCAGTTTCACACTCAACTG	CATGTGATCTATTGTGTACCATCAGG	58	FP	3436	146	323+
L1HS371	AC026611	3	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS372	AC022077.13	3	GAAGAGAAAGAGGAAATAGCACAGG	CTATCCCATAGATGGTGGGTAGAAT	60	IF	1779	599	431+
L1HS373*	AC022838	3	GAAAGAGAGTTCTCTGTACCACACC	GTCATGTCCCAACAGGACATTT	60	VLF	6294	215	231
L1HS374	AC063919	3	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6265	-	-
L1HS375	AC023139	3	TGTGGTACAGTCACACTACAAAG	GATAGCATACACCATCATGCACT	60	IF	3862	430	469+
L1HS376	AC069203	3	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS377	AC078856	3q	GGGAGATGTAGAGTTTTATGTGACC	CTAATGTGCTGGGCAAACATAAGAT	57	FP	577	139	201
L1HS378	AC069225	3	CTCCCCTTTTTGCCTTACTTCT	CTTACTTGCAATAGCCCATTCAC	60	IF	5569	646	369+
L1HS380	AC024470	3	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-

L1HS382	AC055732	3	GCAGACACTAGAAGCTTTTGCAT	GCCACAAAATCTGGCACTTATAG	58	FP	3357	426	185
L1HS383*	AC017085	3	ATTAGTCAGTAATAGAGCCCCCTGT	AAAGACTTCTTTCCAGCTCTACCC	60	FP	6493	267	515
L1HS385	AC078808	3	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6068	-	-
L1HS386	AC023438	UL	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS387	AC069417	3	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS388	AC025818	3	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	713	-	-
L1HS389	AC024216	3	CATGTAGAGATGATCTTCAAAGCTG	GCCTGATAAAAGTAGACACACCTG	60	FP	1782	162	263
L1HS390	AC036128	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS391	AC022040	4	GTGGACATCAGAGTATCCCTTTCT	AGAAGGGTACATGACAACTGGTTAG	60	HF	889	113	203
L1HS393	AC013336	4	TACACAGAATCTGATGCTAGGAGAG	CGGGAACATAAAGTCATAGCGTAAC	61	LF	751	277	412+
L1HS395	AC067804	4	GTTGCATTTTGGAAAGGAAGG	TAGTGGAAAGACAGACAGTTTAGGG	61	IF	1218	119	214
L1HS396	AC007512	4	AGACTCAAACTCAAAACTCCTGTGT	TCACAAGCAGACATTTCTTACTGAA	60	FP	6643	562	373+
L1HS397	AL161439	6	ACTCATCCTAGAGCTTTACCCAGTT	CACAAAGTCAACAGGTTTGATCC	58	FP	1085	259	231+
L1HS398	AC069349	8	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS399	AC027502	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	614	-	-
L1HS401	AC068037	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1342	-	-
L1HS402	AC020593	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	361	-	-
L1HS403@	AL158816	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	360	-	-
L1HS404	AC021700	4	CCACCTTACGTTCAGCTGTTAAT	CGGTGATTAGGTGACAGCTTTT	60	LF	3262	163	231+
L1HS405	AC032017	4	ATCAAAAGTCCTGTGTGTTTGTCTT	GAAATTTTGCTAGACATAGCTGTCC	60	FP	1206	396	202+
L1HS406	AC067842	4	GCAAGTTTTACCCATAGTACACAGG	GTATGTAGAAGGCAGGGGTACACT	60	HF	3589	209	302
L1HS407	AC041010	4	CTCACCAGTACGAGAAGCAAGTT	TCTGACCTAGGGATGATTCTTCA	60	FP	413	227	217
L1HS408	AC019133	4	TTTTAGCCAAGCTCTTTGTTCC	CATTATGGCAGCGTAGACATTG	56	FP	2059	106	209
L1HS409	AC027782	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS410*	AC011633	4	GCTAAGCAATGGAGGAAAATATCG	TGTACATGGTGTGAGGTATGAA	57	IF	6211	100	244+
L1HS411	AC073338	4	ACACACACACGATGGAAAGTATCT	AGCACATCCTAAATCTTCCTCTCT	60	FP	2670	136	246
L1HS412	AC067901	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS413*	AC023332	4	TCATGAGCATCACTCTTACCATGT	ACTCAGCTGACTTGCCATAAATGT	60	IF	6199	127	191
L1HS414	AC025955	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS415	AC009816	4	TCAGACCCATATATGAGCATAACC	GCTTAGAAGAATTTTTAGCCAGGTG	56	HF	1360	590	476+
L1HS416	AC068256	4	TTAGTCACTATGACTTGAGCCACTT	TAGTGATAGTGTAGAGAGGGGGTTG	61	FP	822	238	284
L1HS417	AP001860	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	865	-	-
L1HS418*	AC011981	2	CGATTTCTGTCTTTGTGAACGTAGT	CCTTACAGAGTAGAAATCTCACGAT	60	IF	6380	328	358
L1HS419	AC061978	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6034	-	-
L1HS420	AC041038	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6066	-	-
L1HS421	AC024974	UL	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS422	AC009577	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS423	AC022672	11	CTCCCTGTCTTCTGGGTTAAAATA	GGAAGTCCCACTTTTTCAGTAGAG	60	HF	5680	201	248+
L1HS424	AC080124	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS425	AC013724	4	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6120	-	-

L1HS426	AC023921	5	AGATTCCCTTTGGTATCCAAATCAC	GTTGCCATACTCCGCATAAAGTC	60	IF	3394	204	252
L1HS427	AC015990	4	TACGGGCAAAGACTGAGAGTACTAA	TTCAGCCTTCTGACATCAAACT	57	IF	2230	139	220+
L1HS429	AC060816	4	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS430	AC024963	4	CAGAGAACCAACATGTAGGAACAA	GTTACAGGTCAAAGGAGGTCTGAG	60	LF	4034	127	223+
L1HS432	AC011399	5	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS433	AC027339	5	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS434	AC010437	5	ACCTGGGCCACATTTATTTTTC	TGTAGAAGAAGACACCGTCGTTAG	60	FP	2637	250	246+
L1HS435	AC026403	5	GACTCAGTTGACAAGGAGTTACCA	ACACTAGCTTCTTTGACTTCACCA	55	FP	1115	111	211+
L1HS437	AC023526	5	ATCTATCATTTATCTGCCCCGTCT	ACAAGGATTAGCAGGAAGTCTGTT	60	IF	2954	256	201+
L1HS438	AC011433	5	TCCTCTCACCAACCACATAAAGTA	ATCCCTTGGATACAAAGATGTGC	60	FP	1909	570	345
L1HS439	AC016573	5	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS440	AC010409	5	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6133	-	-
L1HS441	AC026444	5	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS442	AC027325	5	GACGGTTACTCAGAAAAACACAAG	GTAGATGCCACTGTTACCCTGACT	60	IF	907	224	185+
L1HS443	AC021600	5	GCTAGACTCTCTACCTTTGGCTTT	TGATACCTGACTCTATGCACCACT	56	FP	891	261	382
L1HS444	AC027315	5	TTATTGGAATAGCTTCTCCTGTCAC	GCTGTTCCTAACTCTAGTCCTCCA	60	FP	464	303	296+
L1HS445	AC008374	5	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	551	-	-
L1HS446	AC010314	5	CTCGTGACATTTCCATCATATAGC	TTAAGTCACCTAAGGGTTGTAAGTG	56	LF	6142	109	182+
L1HS447	AC018759	5	GTACATCTCTTTGGACACTTCCACT	GTTTAAGTCCAACATCCTGTTCTG	59	IF	691	560	386
L1HS448	AC016545	5	GTCAATTAGAGCATGAAGAAACCAC	GTACATCTCTTTGGACACTTCCACT	60	IF	652	525	382+
L1HS449	AC011378	5	CTAGGGAGGTGAAAATTCAGATGT	GCATGTTGCACAACAGTATGTA	60	FP	1797	281	315+
L1HS450	AC011413	5	GTGAAGACTGTTGGTCAGTTACTTGT	GTCATTGAGATTGGCAGGTAAAAG	60	HF	6179	128	189+
L1HS451	AC010490	5	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	994	-	-
L1HS453	AL360232	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6064	-	-
L1HS455	AC027643	6	CATACACAAGGGCGAAGAGTTAAA	GCCTCTTTTACATCAGTTACCACTC	60	FP	259	110	213+
L1HS456	AC026966	6	TAACACTTAGTGATTGCTGGGAGAG	GGACAAGGTGAAGTGGAAAACTAGA	60	FP	1641	121	215
L1HS457	AC025887	18	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	286	-	-
L1HS460	AL355489	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6044	-	-
L1HS461	AL358992	6	ATCCAGCAAAAGTATCCCTTAAGTA	TCCTGTCCCAATTCTTTGTATTAT	60	LF	4143	324	417
L1HS462	AC069403	11	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	4163	-	-
L1HS463	AL391336	6	ATTAAATCTGTGTGGGAGTGG	AGGGTGACTTCAGTGATATCTTCA	60	FP	6304	247	346
L1HS465	AL356601	6	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1936	-	-
L1HS469@	AC020586	UL	GGTACTGGCTGTTCAGTATTTTT	GTCTCAAAGCCCATTTCATAGTTC	60	FP	6458	101	212+
L1HS472	AC018400	UL	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS476	AC079756	7	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	897	-	-
L1HS477	AC024730	7	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1271	-	-
L1HS478	AC069008	7	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	991	-	-
L1HS479	AC079855	7	CACTCGAAGGGTAAGTGAGATTTT	CCACTAGCGCACCATTTTTCTAAT	58	FP	6223	146	276
L1HS480	AC021836	4	AGAGGTAACCACTACCTTGCAACT	GCCTCATGACAGGAGAAGAGATAAA	60	IF	2701	272	265
L1HS483	AC026011	8	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-

L1HS484*	AC073647	7	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6692	-	-
L1HS485	AC027189	8	CTCAGTTCCACATAAACCTTGACA	GAAGCAATTAACCTAGCAGTAGGAC	60	FP	548	74	183+
L1HS486	AL356516	9	CCCTCATCACCAAATATCTGAGAA	AGCTGACAGTCTAGTGAATGAGGTC	60	IF	905	139	196
L1HS487	AL162731	9	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6079	-	-
L1HS488*	AL353649	9	CAAATTGTCAATGCTAACCACTCC	GGAAAAAGGCACTTTGGCTTATC	62	FP	6787	724	472+
L1HS489	AC009284.2	9	TCTCCAGAAACCATCACAGTAAGA	AGGAGTTGAAAGTAGGATGGGTTT	60	FP	322	104	202+
L1HS490@	AL358937	9	CAGCTGTCTTGCTAAGAATCCAT	AGACCACAGACTCTTTGAGGGTAAG	60	FP	2289	397	206
L1HS491	AL355303	10	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS492	AL450466	10	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS493	AL138764	10	GACTACCTTTCTGCGTATTCCTTTC	GTCTAACAGGTACACGAGACTCCAT	61	IF	1603	111	241+
L1HS494	AC068972	8	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2974	-	-
L1HS495	AC083848	8	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1341	-	-
L1HS496	AC024929	8	CCTTTGGAAGAGAAAGAGGATATG	CTCCCAATGGAAAGGAACTTGTAT	60	FP	617	70	177
L1HS497	AC060775	8	GCCTAGTGGGAAGACAAAAAGTATT	GCTGTAATGTTAACCTCGAAGTCGT	60	FP	950	346	439+
L1HS498*	AC067844.3	8	AGGTTTCCCCAAAATTTACCC	CTGATGTGTGGATTCACTGTTCTT	58	FP	6281	184	295
L1HS499	AC024649	8	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1045	-	-
L1HS500	AC009630.5	8	GTGTTGCCTTCACCACAATAGTA	TTTCTCCGAGTACAGGTTACGAG	60	FP	1145	206	227+
L1HS501	AC022207	12	GTTGGCAACTTACTCTCAAATGG	AAATACACTCGACTGGCCACTAA	60	FP	6254	199	306+
L1HS502	AC011881	UL	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	537	-	-
L1HS503	AC055118	13	GTGAGGAATGGTTGAACAGCTT	TGTGGCTGGAGAAATACCTCTAA	60	FP	713	101	206+
L1HS504	AL158045	13	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS505	AL162716	13	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	384	-	-
L1HS506	AL138684	13	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS507	AC064832	15	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS508	AC048381	15	ACAGAACCTTTTAGAGGGAATCG	CTCCGTGTGGTAAAATTAGCTGT	58	HF	6144	103	184
L1HS509	AL356017	14	CACTCATGACTGCCTGACTTCT	CAGGGATTACTCTTCTGTTGTGG	61	FP	443	131	220+
L1HS510	AL390800	14	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1837	-	-
L1HS511	AL162632	14	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6088	-	-
L1HS512	AC021839	14	AAAGAGACAATCCACAGCATAGTTG	GATTTATTCCTTCATGGAGATGTGC	61	HF	2071	722	266+
L1HS513	AL160156	13	CCAAACTTGAGCCTCCTGTAATC	CCTTGAAATAAGCAGGAAGAAGC	61	IF	809	142	235+
L1HS514	AL138961	13	CCTCAGCTTTGGATCCTGTAGTT	AGAAGAATTGGGTCCTGTTGAA	60	FP	6670	334	361
L1HS515	AL163537	13	GGATGGTAAAGGAGTGGCATAAT	TGTGGAGCCCAGATCTTTTAAT	60	FP	637	106	193
L1HS516	AC044907	15	CCACAGTTTACACAGAAGCTGAA	GAAGGAGTGGATGTGTTTCAGTAA	60	IF	6151	101	212
L1HS518	AC074236	15	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2636	-	-
L1HS519	AC074100	15	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS520*	AC015558	15	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6087	-	-
L1HS521@	AC067951	15	GCTTTGTTTACCTTTCTGCTCACT	CACCAAAAGGAGAAGCCAATAAAG	60	FP	1248	344	441+
L1HS522	AC009555	15	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	190	-	-
L1HS523	AC009658.6	15	CGTGGAAGATGTTACGAGGATTA	AGAGAATGCGATGTCGATTAGAG	60	FP	570	105	204
L1HS524	AC020892	15	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-

L1HS525	AC009057	16	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS526*	AC025289	16	ACCCTCCAAGGTAACTGAATCTTA	ATGCCCATGCTTGTTAGCTACTAC	60	IF	6076	223	324+
L1HS527	AC026472	16	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1224	-	-
L1HS528	AC009021.4	16	CGGATGGGAGCACAAAATTACTA	TGCCTACTAAGATACCTTGGAAATG	61	FP	991	172	278
L1HS529	AC022164	16	TGAGTAATGTGGCGGTTTAGTTC	AACCAGTCAAGAAGCCAAAGAG	61	FP	6143	116	193+
L1HS530	AC009063	16	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS531	AC055852	17	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	2839	-	-
L1HS532	AL356138	20	CCTCTAATCTATGGTGGATGCTCT	TGGTAGGGAGCTGGTAAAAGTCTA	61	FP	308	175	242+
L1HS534	AC007448	17	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS535	AC034266	17	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS539	AC034266	17	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS541	AC068204	18	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS542	AC023983	18	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS543	AC009267	18	TACATTAGTCTGCCTCTGATTCCA	GGCCATTCTTTTCATCTGTTGTAG	61	FP	547	99	183
L1HS545	AC007768	18	TGGGAACTCATGTTACAGTTTCAC	ATTTGTCATGATCACAGCCACCT	59	FP	2514	95	216
L1HS546	AP001460	18	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS547	AC010966	18	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS548	AP001113	18	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6237	-	-
L1HS551	AC021325	18	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	184	-	-
L1HS552	AP001564	18	CAGTGAACTGCTTTCTCACAATTC	CAAGAAGTTTTCCTGGAGTCTCTC	60	IF	4144	123	235
L1HS554	AC027230	18	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	561	-	-
L1HS556	AC026898	18	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS557	AP001019	18	ACAAAAGCACCTAGAAGCAGTCAT	CTTTTTCTCCTATGCTCGTGGTAT	60	FP	2277	85	229+
L1HS558	AC015819	18	TGCTTTCTTTCTTTCACATAGATCA	GCAGACACGAATCACAGTTTGTAT	61	HF	983	128	203+
L1HS559	AC023394	18	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1620	-	-
L1HS561	AC013620	14	TACCCATTTAAAGGGCAAAGTG	CTACCCATTTAAACCACTAATGCTG	61	LF	430	114	239+
L1HS562*	AC019175	X	TGTCTGTTCAGTCCTTTCTCACAT	AGCAAAATGTATGCCGAAGACT	59	FP	6170	115	181
L1HS564	AC034155.5	X	TGCAATTGACATAGATACTGCAGAG	CCCTTCCCTTTCTGTACATGTCTT	61	LF	2085	471	425+
L1HS565	AL442646	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	6029	-	-
L1HS567	AL158143	X	END OF SEQUENCING CONTIG	END OF SEQUENCING CONTIG	-	EC	-	-	-
L1HS568	AL356003	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	1297	-	-
L1HS569	AC021992	X	INSERTED IN REPEATS	INSERTED IN REPEATS	-	R	596	-	-

* Full length elements with intact open reading frames.
# Elements previously identified by Boissinot et.al. 2000
@ Elements previously identified by Sheen et.al. 2000 and Ovchinnikov et.al. 2001

1. Chromosomal location was determined from Accession information or by PCR analysis of NIGMS monochromosomal hybrid cell line DNA samples.
2. Amplification of each locus required 2:30 min @ 94°C initial denaturing, and 32 cycles for 1 min 94°C, 1 min Annealing Temperature (A.T.), and 1 min elongation at 72°C. A final extension time of 10 min at 72°C was also used.
3. Elements at the end of sequencing contigs are denoted (EC), those residing in other repeats (R), those having paralogs (Paralogs), and elements with inconclusive PCR results (NR). Elements represented here are classified according to allele frequency as: high frequency (HF), intermediate (IF), low (LF), very low (VLF) or "private" insertion polymorphisms, or as fixed present (FP) insertions. Fixed present: every individual tested had the LINE element in

both chromosomes. Low frequency insertion polymorphism: the element is present in no more than 1/3 (33%) alleles tested. Intermediate frequency insertion polymorphism: the element present in more than 1/3 (33%) of alleles tested and no more than 2/3 (67%) of the alleles. High frequency insertion polymorphism: the element is present in more than 2/3 or 67% but not all alleles tested. Indeterminable data is denoted (-).

4. PCR Product Sizes: Empty product size is calculated computationally by removing the L1Hs Ta elements and 1 direct repeat from identified filled site. Subfamily Specific product size is calculated from internal subfamily specific primer located in the 3' UTR to the proximal 3' primer. In cases where target site duplication sequence were not found flanking the element PCR product sizes may vary from those reported. Elements with subfamily product size denoted "+" were found in 5′→3′ orientation in GenBank and are assayed using the internal subfamily specific primer and flanking reverse primer. All other elements were assayed using the internal subfamily specific primer and flanking forward primer.

Increased Frequency of Pre-germinal Center B Cells and Plasma Cell Precursors in the Blood of Children with Systemic Lupus Erythematosus[1]

Edsel Arce,*† Deborah G. Jackson,*† Michelle A. Gill,*† Lynda B. Bennett,*† Jacques Banchereau,* and Virginia Pascual[2]*†

We have analyzed the blood B cell subpopulations of children with systemic lupus erythematosus (SLE) and healthy controls. We found that the normal recirculating mature B cell pool is composed of four subsets: conventional naive and memory B cells, a novel B cell subset with pregerminal center phenotype ($IgD^+CD38^+centerin^+$), and a plasma cell precursor subset ($CD20^-CD19^{+/low}CD27^{+/++}$ $CD38^{++}$). In SLE patients, naive and memory B cells ($CD20^+CD38^-$) are ~90% reduced, whereas oligoclonal plasma cell precursors are 3-fold expanded, independently of disease activity and modality of therapy. Pre-germinal center cells in SLE are decreased to a lesser extent than conventional B cells, and therefore represent the predominant blood B cell subset in a number of patients. Thus, SLE is associated with major blood B cell subset alterations. ***The Journal of Immunology,*** **2001, 167: 2361–2369.**

Lymphocyte counts are known to be significantly decreased in systemic lupus erythematosus (SLE)[3] and lymphopenia of <1500 cells/μl is the most prevalent initial laboratory abnormality in this disease (3). Despite the low circulating lymphocyte levels, B cells play a major role in the pathogenesis of SLE in both humans and murine SLE models, as they are responsible for the hypergammaglobulinemia and autoantibody production that characterize this disease (4, 5). Most studies on lupus B cells have been performed on mice with lupus-like syndromes (6–9) rather than human SLE (10–14). Interestingly, MRL/*lpr* mice expressing surface Ig but lacking secreted Ig develop nephritis, suggesting that B cells may play a role in the pathogenesis of SLE nephritis that is independent from serum autoantibodies (15). With regard to humans, SLE B cells exhibit, upon signaling through the Ag receptor, increased Ca^{2+} flux and early protein tyrosine phosphorylation (12). SLE B cells express high levels of costimulatory molecules CD80 and CD86 (13) as well as CD40 ligand (CD40L)/CD154 (14). High levels of soluble CD40L are also found in the serum of active SLE patients (16, 17).

In recent years our laboratory has developed methods to isolate and characterize mature peripheral B cells. Using anti-IgD and anti-CD38 Abs, four mutually exclusive peripheral B cell populations can be isolated (reviewed in Refs. 18 and 19). Single-positive IgD cells correspond to follicular mantle cells (Bm1 + Bm2), whereas single-positive CD38 cells correspond to germinal center (GC) cells (Bm3 + Bm4). Double-negative B cells correspond to the memory population (Bm5), whereas double-positive cells represent a combination of cells at a transitional stage between follicular mantle and GC (Bm2′) and single-isotype IgD^+ GC cells (20). More recently, CD27 has been reported as marker of memory B cells within both the $sIgD^+$ and $sIgD^-$ peripheral B cell compartments (21, 22). The phenotypic summary of these populations is depicted in Table I.

These studies and those by others (23–30) have led to the proposal of a model of T cell-dependent, Ag-dependent mature B cell differentiation: naive B cells (Bm1 and Bm2) are activated in association with Ag-specific T cells and interdigitating cells within the extrafollicular areas. The activated B cell blasts either undergo terminal differentiation toward plasma cells (extrafollicular reaction) or become GC founder cells (Bm2′). In GCs, Bm2′ differentiate into centroblasts (Bm3) that proliferate and accumulate point mutations into the Ig variable region genes, yielding three types of mutants: high affinity, low affinity, and autoreactive mutants. These mutants will be selected while they differentiate into centrocytes (Bm4), their survival depending on their affinity for the Ag trapped within immune complexes bound to follicular dendritic cells. The high affinity mutants will pick up the Ag, process it, and present it to GC T cells, which are induced to express CD40L and secrete cytokines (i.e., IL-4 and IL-10), key elements for survival, proliferation, and isotype switching. These high affinity centrocytes differentiate into either memory B cells (Bm5) or plasma cells. Low affinity mutants that do not bind FDC-bound Ag will die by apoptosis, whereas autoreactive mutants are eventually deleted because they do not receive T cell help. During secondary humoral immune responses, recirculating memory B cells can be activated in extrafollicular areas, giving rise to plasma cells and GC founder cells.

Although extensive information has accumulated on the mature B cells that populate peripheral lymphoid organs such as human tonsils, little is known about blood B cell subsets. We have thus

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[3] Abbreviations used in this paper: SLE, systemic lupus erythematosus; CD40L, CD40 ligand; GC, germinal center; JDM, juvenile dermatomyositis; SLEDAI, SLE disease activity index.

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Table I. *Surface marker expression of mature human B cell subpopulations*

	Pan-B		Naive			GC				Memory	Plasma Cell
Surface Markers	CD19	CD20	sIgM	sIgD	CD23	CD38	CD10	CD71	CD77	CD27	CD138
Naive (Bm1 + Bm2)	+	+	+	+	−/+	−	−	−	−	−	−
GC Founder (Bm2′)	+	+/++	+	+	−	+	+	+	+	+	−
GC (Bm3 + Bm4)	+	++	+/−	+/−	−	+	+	+	+	+	−
Memory[a]	+	+	+	+	−	−	−	−	−	+	−
Memory (Bm5)	+	+	+/−	+/−	−	−	−	−	−	+	−
Plasma cell	Dim	−	−	−	−	++	−	−	−	+/++	+

[a] $sIgD^+sIgM^+$ memory cells.

analyzed the peripheral blood B cell compartment of healthy adults, healthy children, and children suffering from rheumatic diseases including juvenile dermatomyositis (JDM) and, most particularly, SLE. These studies have permitted us to identify a novel blood B cell population expressing a partial GC phenotype and an oligoclonal plasmablast population. Although these populations are not restricted to SLE patients, the disproportionate depletion of conventional naive and memory B cells in SLE make pre-GC cells and plasmablasts predominate in SLE blood.

Materials and Methods

Samples and patient populations

Blood samples from 35 healthy children, 68 children with SLE, 10 with JDM, and 17 healthy adults were drawn after informed consent in accordance with our institutional internal review board was obtained. All pediatric SLE patients included in this study fulfil the established American College of Rheumatology criteria for SLE (31). The patients' clinical and serological data were gathered during clinic visits, and the corresponding SLE disease activity index (SLEDAI) was recorded in the chart (32). The average ± SD age and the sex ratio for each of the groups were: 1) healthy children group, 12.15 ± 3.15 years, 3:1 female/male; 2) pediatric SLE group with SLEDAI >10 (n = 36), 14 ± 2.67 years, 5:1 female/male; 3) pediatric SLE group with SLEDAI <10 (n = 32), 13 ± 3.15 years, 6:1 female/male; 4) JDM group, 9.2 ± 3.8 years, 4:1 female/male; and 5) adult group, 36.8 ± 6.21 years, 3:2 female/male. SLE patients belong to different ethnic backgrounds, including Caucasian (32.3%), African-American (25.3%), Hispanic (23.9%), and Oriental (4.2%). The healthy children control group had a similar ethnic distribution. Therapy guidelines for childhood SLE are similar to those for adult SLE patients. Most of the included patients were being treated with oral prednisone and hydroxychloroquine, and those with type III/IV nephritis and/or major extrarenal organ involvement were receiving i.v. cyclophosphamide (~20% of patients) and/or methylprednisolone (~40% of patients). Blood samples were drawn at least 4 wk after the last i.v. pulse of either of these medications had been administered. Selected patients with JDM had active disease and were treated with oral prednisone and/or i.v. methylprednisolone at doses comparable to those given the SLE patients (10/10).

Flow cytometric analysis of blood B cells

Two methods have been used to assess blood B cells. The first analyzes purified B cells, whereas the second analyzes total blood and has the considerable advantage of necessitating only 0.5 ml (rather than 10–20 ml) of blood. Samples from 44 SLE patients, 22 healthy children, 10 JDM, and 17 healthy adults were analyzed using enriched B cells, whereas samples from 24 SLE patients and 13 healthy children were assessed using whole blood. The validity of the whole blood method has been established on three patients and yielded comparable results, therefore permitting us to pool the results of a 30-mo-long study. Absolute numbers of cells were calculated from the relative size of total B cells and B cell subpopulations and the absolute leukocyte and/or PBMC counts.

Isolation of peripheral blood B cells

Mononuclear cells were isolated using gradient centrifugation over a Hystopaque cushion. The resulting population was enriched for B cells using negative depletion with magnetic beads coupled to anti-CD2, CD3, CD4, CD14, CD16, CD56, and glycophorin A (stem cell). The enriched B cells were stained with fluorochrome-labeled Abs (FITC, PE, Tricolor, PerCP, and allophycocyanin). The following were used: anti-human CD3-FITC, CD7-FITC, CD14-PE, CD19-allophycocyanin, CD20-PerCP (BD Biosciences, Mountain View, CA); CD10-FITC, CD40-PE, CD71-FITC, CD79a-FITC (Immunotech Research, Quebec, Canada); CD23-PE, CD56-FITC (Caltag, South San Francisco, CA); CD38-PE, CD5-PE, CD138-FITC, κ and λ light chain-PE (Serotec, Oxford, U.K.); CD154-FITC (Ancell, Bayport, MN); and anti-human IgD-FITC, IgM-PE, IgG-PE, IgE-FITC, IgA-FITC (Southern Biotechnology Associates, Birmingham, AL). Stained cells were analyzed using flow cytometry (FACSCalibur, BD Biosciences). All experiments were analyzed after gating on live cells according to forward side scatter/side light scatter. A minimum of 100,000 cells was used for each staining condition, and 5,000–50,000 events were recorded for analysis. Selected populations of cells were sorted for immunohistochemistry or molecular studies using the FACSVantage (BD Biosciences) instrument.

Labeling of cell surface Ags from whole blood samples

Whole blood was collected into tubes containing heparin or ACD and stained with the following Abs: IgD-FITC,CD38-PE, CD20-PerCP, and CD19-allophycocyanin and corresponding isotype controls. We used 50 μl blood and 3 μl of each Ab per tube for each staining. After staining, the blood was lysed with FACS Lysing Solution (BD Biosciences), rinsed with PBS, centrifuged at 1200 rpm for 10 min, and resuspended in 1% paraformaldehyde. Samples were then analyzed on a BD Biosciences flow cytometer (FACSCalibur).

Amplification of the centerin gene

Real-time PCR was performed using an ABI Prism 7700 sequence detector (PE Biosystems, Foster City, CA). The RT-PCR conditions were 30 min at 48°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. The Taqman PCR core kit reagents (PE Biosystems), Multiscribe reverse transcriptase (PE Biosystems), and RNase inhibitor (PE Biosystems) were used according to the manufacturer's suggested concentrations for a multiplex reaction. The 18S ribosomal RNA and Centerin standard curves were generated using a serial dilution of a known quantity of Raji total RNA. Ribosomal RNA analysis was performed using the ribosomal RNA control reagent kit (PE Biosystems). The centerin probe (6-FAM-tcaccagaaccatggccgtcagaag-TAMRA) was used at a concentration of 250 nM, and the forward and reverse centerin primers (forward aagggaaggtt gtagacataatcca; reverse gcttctcccacttggctttaaa) were used at a concentration of 900 nM.

Sequencing of Ig V_H genes

Total RNA from between 1,000 and 100,000 sorted B cells was prepared using the mini-RNEASY kit, (Qiagen, Valencia, CA) following the manufacturer's protocol. RT-PCR was performed on 10% of the total RNA generated from the sorted cells using the Titan RT-PCR kit (Roche, Indianapolis, IN). The V_H region of IgM transcripts was amplified using either a V_H4 or a V_H5 leader primer in combination with a μ-constant region reverse primer, as previously described (33, 34). The V_H region of IgG was amplified using identical forward primers with a γ-specific constant region reverse primer. The V_H fragments were excised from a low melt agarose gel and reamplified using heminested reverse primers and the high fidelity PFU polymerase (Stratagene, La Jolla, CA). The PCR fragments were either *t*-tailed with *Taq* polymerase (Promega, Madison, WI) and subsequently cloned into the pCRII-TOPO vector or directly cloned into the pCR-blunt-II-TOPO vector (Invitrogen, Carlsbad, CA) and sequenced in both directions using an automated DNA sequencer (ABI-377; Advanced Biotechnologies, Columbia, MD).

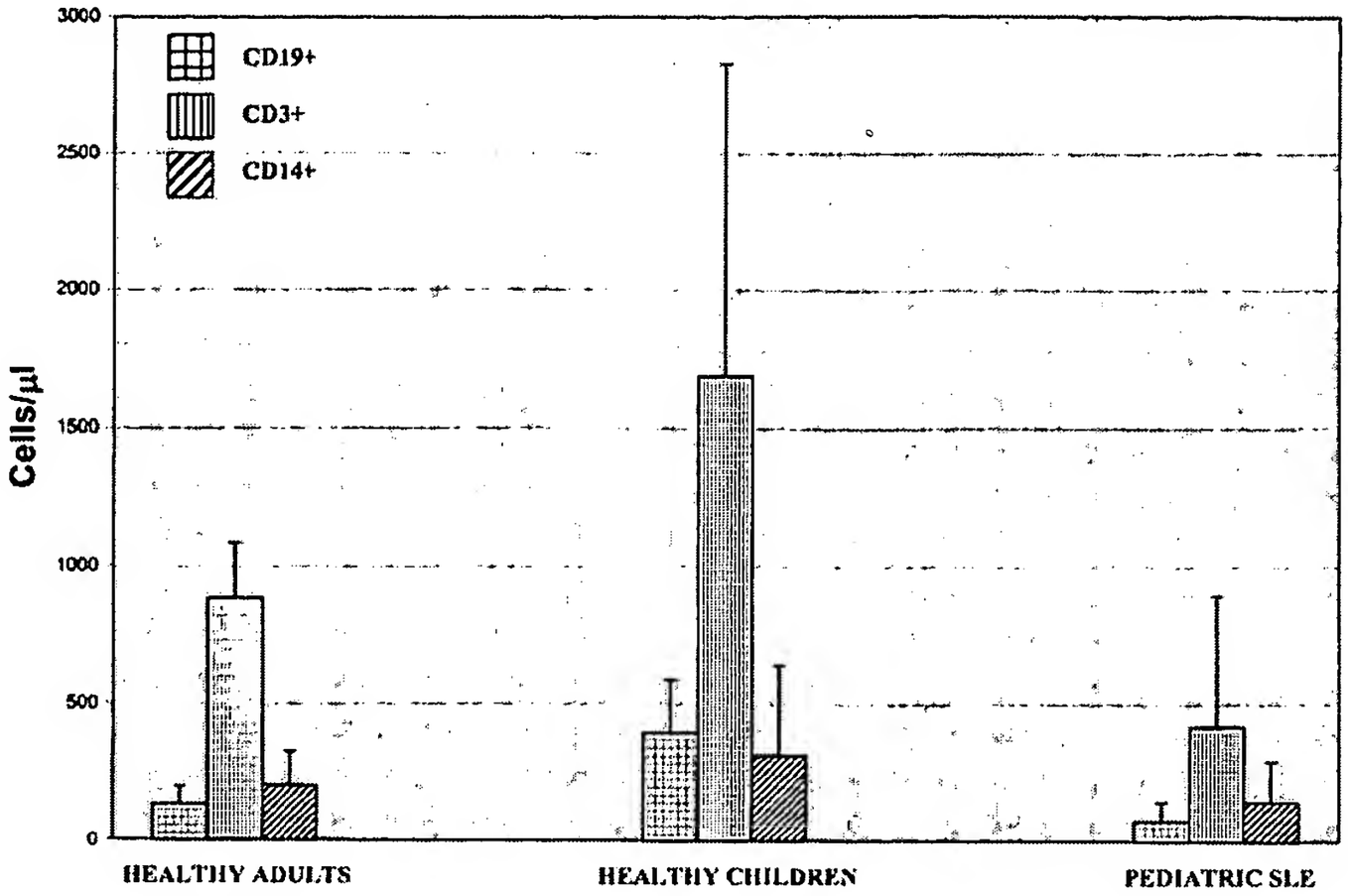

FIGURE 1. Mean and SD of blood B cell, T cell, and monocyte numbers from healthy adults, healthy children, and children with SLE.

Analyses of DNA sequences

Sequences were edited and analyzed using the DNAstar software package (DNAstar, Madison, WI). Cloned products were searched against the IMGT (the international ImMunoGeneTics database, http://imgt.cines.fr: 8104) (35).

Statistical analysis

The data obtained in this study were evaluated using a two-tailed *t* test and multivariable statistical analysis, as well as the Pearson correlation ratio.

Results

T and B cells are profoundly decreased in SLE blood

Although lymphopenia has been described in SLE (3), the extent of T and B cell decrease remains uncharacterized. Therefore, we measured the absolute numbers of $CD3^+$, $CD14^+$, and $CD20^+/19^+$ cells in the blood of 1) 68 children suffering from SLE, 2) 35 age-matched healthy controls, 3) 10 children with JDM to control for the effect of steroid treatment, and 4) 17 healthy adults. SLE patients were divided into two groups according to their disease activity index (SLEDAI over or under 10) measured at the time of blood sampling. The ages (mean and SD) of the SLE patients and healthy controls were comparable (see *Materials and Methods*). As previously reported (36, 37), when compared with adults healthy children display significantly more blood $CD3^+$ T cells (1687 ± 1139 vs 881 ± 202 cells/μl; $p = 0.002$) and $CD19^+$ B cells (394 ± 196 vs 129 ± 67 cells/μl; $p < 0.0001$; Fig. 1). Children with JDM, treated with steroid regimens similar to those of SLE patients, display numbers of $CD19^+$ cells comparable to those in healthy controls (Table II and Fig. 2). The slight difference (not statistically significant) may reflect the lower average age of the JDM group (9.2 ± 3.8 vs 12.1 ± 3.5 years in JDM and healthy controls, respectively).

Children with SLE showed significantly fewer circulating T cells than healthy children (450 ± 300 vs 1700 ± 380 cells/μl; $p < 0.0001$). Although patients with the highest disease activity (SLEDAI, >10) had lower numbers of T cells than patients with lower disease activity (SLEDAI, <10; 310 ± 167 vs 510 ± 467 cells/μl), this difference was not statistically significant. SLE patients had fewer circulating monocytes than healthy children (144 ± 149 vs 313 ± 326 cells/μl), but this difference did not reach statistical significance ($p = 0.06$; Fig. 1).

Blood $CD19^+$ B cells in SLE patients were reduced by 81% compared with those in age-matched healthy controls (82.6 ± 77.5 vs 394 ± 196 cells/μl; $p < 0.0001$). There was no difference in the number of circulating B cells between the two patient groups (Table II), suggesting that B cell lymphopenia in SLE is independent of disease activity. Although most of our patients had been treated for weeks to years with steroids at the time of study, the T and B cell lymphopenia is not a consequence of this therapy, as newly diagnosed patients (3 of 68) were also found to have similarly decreased numbers of T and B cells before they had entered into

Table II. *Mean and SD numbers of cells per microliter in each of the studied populations of healthy donors and patients*[a]

Sample (*n*)	Total $CD19^+$ Mean ± SD	$CD19^+CD20^+CD38^-$ Mean ± SD	$CD19^+CD20^+CD38^+$ Mean ± SD	$CD19^+CD20^-CD38^{++}$ Mean ± SD
Healthy adults (17)	129.6 ± 67.5[b]	97.7 ± 49.7[c]	18.1 ± 18.7[d]	1.4 ± 1.7[c]
Healthy children (35)	394.0 ± 196.1[c]	270.9 ± 157.9[c]	57.8 ± 59.3[c]	6.3 ± 9.2[e]
JDM (10)	470.7 ± 298.4[c]	428.4 ± 294.6[c]	37.4 ± 31.2[d]	4.2 ± 5.5[c]
Total SLE (68)	82.6 ± 77.5	28.0 ± 40.3	19.9 ± 24.5	18.7 ± 22.2
SLEDAI >10 (36)	75.2 ± 81.1	28.6 ± 40.2	21.4 ± 27.7	19.5 ± 12.8
SLEDAI <10 (32)	90.5 ± 74.0	27.1 ± 41.6	18.2 ± 20.6	18.0 ± 19.9

[a] Superscript letters indicate the statistical significance between control and SLE groups.
[b] $p = 0.006$.
[c] $p < 0.001$.
[d] NS.
[e] $p = 0.001$.

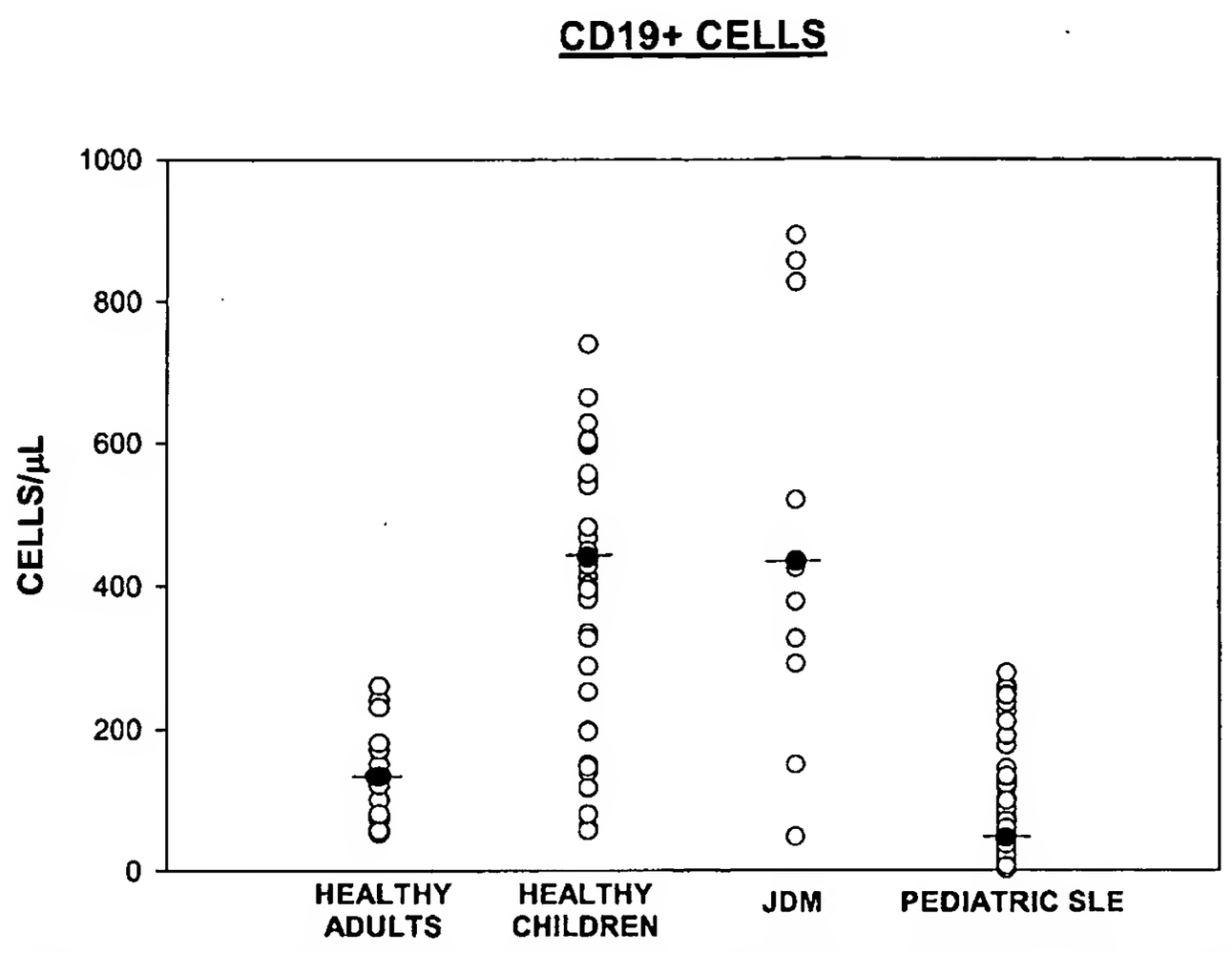

FIGURE 2. Blood B cell and plasma cell precursor ($CD19^+$) numbers in SLE patients and controls. ●, Median values.

any therapy (64.2 ± 72.1 B cells/μl; $n = 3$). Additionally, nine of the patients treated with i.v. solumedrol and cyclophosphamide who were included in this study have been followed after discontinuation of these drugs for periods between 6 mo and 2 years without finding statistically significant differences in the number of B cells (data not shown).

Circulating naive and memory B cells are considerably reduced in SLE

Our earlier studies on tonsillar B cells showed that CD38 expression permits us to distinguish plasmablasts/plasma cells and GC B cells from naive and memory B cells (reviewed in Refs. 18 and 19). Thus, $CD19^+CD20^+CD38^-$ blood cells include both naive and memory B cells. As shown in Table II, healthy children displayed significantly more conventional mature ($CD19^+CD20^+CD38^-$) B cells than adults (270 ± 157 vs 97 ± 49 cells/μl; $p < 0.0001$). In contrast, SLE patients showed a marked reduction (~90%) in these cells compared with age-matched controls (28.0 ± 40.3 cells/μl; $p < 0.0001$). This reduction does not appear to be related to disease activity (27.1 ± 41.6 cells/μl for SLEDAI <10; 28.6 ± 40.2 cells/μl for SLEDAI >10; Table II).

The blood memory B cell population is best identified as $CD20^+CD27^+$ cells. We calculated the ratio of memory/naive B cells in healthy children and children with SLE and found no difference between the two groups (0.46 ± 0.30 and 0.49 ± 0.35 in healthy and SLE children, respectively).

B cells with pre-GC phenotype recirculate in blood of healthy and SLE children

Our initial studies on SLE total blood and enriched blood B cells revealed a strikingly high percentage of circulating $CD20^+IgD^+CD38^+$ cells. A closer analysis of samples from non-SLE patients revealed that cells with similar phenotype were also present in the blood of healthy children, adults, and children with autoimmune diseases other than SLE, prompting us to report their characterization (Fig. 3). In absolute numbers healthy children have the highest numbers of IgD^+CD38^+ cells (57.6 ± 53.3 cells/μl), followed by patients with JDM (37.4 ± 31.2 cells/μl). The number of IgD^+CD38^+ cells in SLE patients (21.4 ± 27.7 cells/μl SLEDAI >10, 18.2 ± 20.6 cells/μl SLEDAI <10) is comparable to that in adults (18.1 ± 18.7 cells/μl; Table II). Due to the more drastic reduction in conventional $CD20^+CD38^-$ cells in SLE patients, this population overall represents 29 ± 17.7% of SLE blood B cells (range, 6–77%), whereas it represents 13.2 ± 8 and 18.5 ± 14.9% of the total blood B cells in healthy adults and children, respectively (Fig. 4).

In both patients and controls these cells express high CD20, a characteristic of GC B cells (data not shown). When sorted and analyzed with Giemsa staining, IgD^+CD38^+ cells appear very

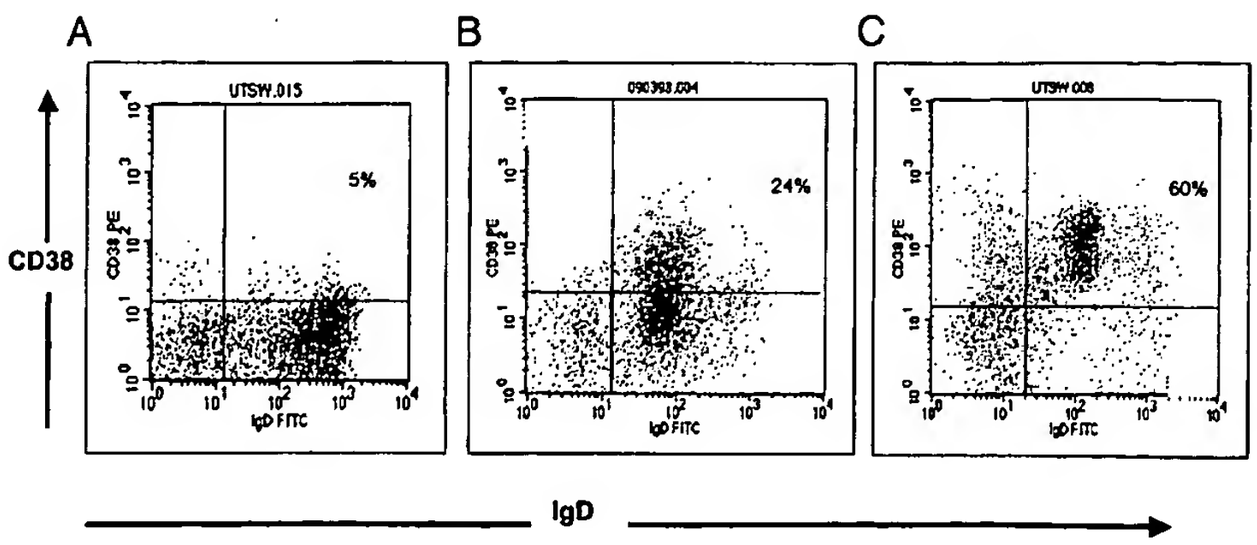

FIGURE 3. Enriched blood B cells from a healthy adult (*a*), healthy child (*b*), and a child with SLE (*c*) stained with anti-CD38-PE and anti-IgD-FITC Abs. Double-positive cells display a pre-GC phenotype.

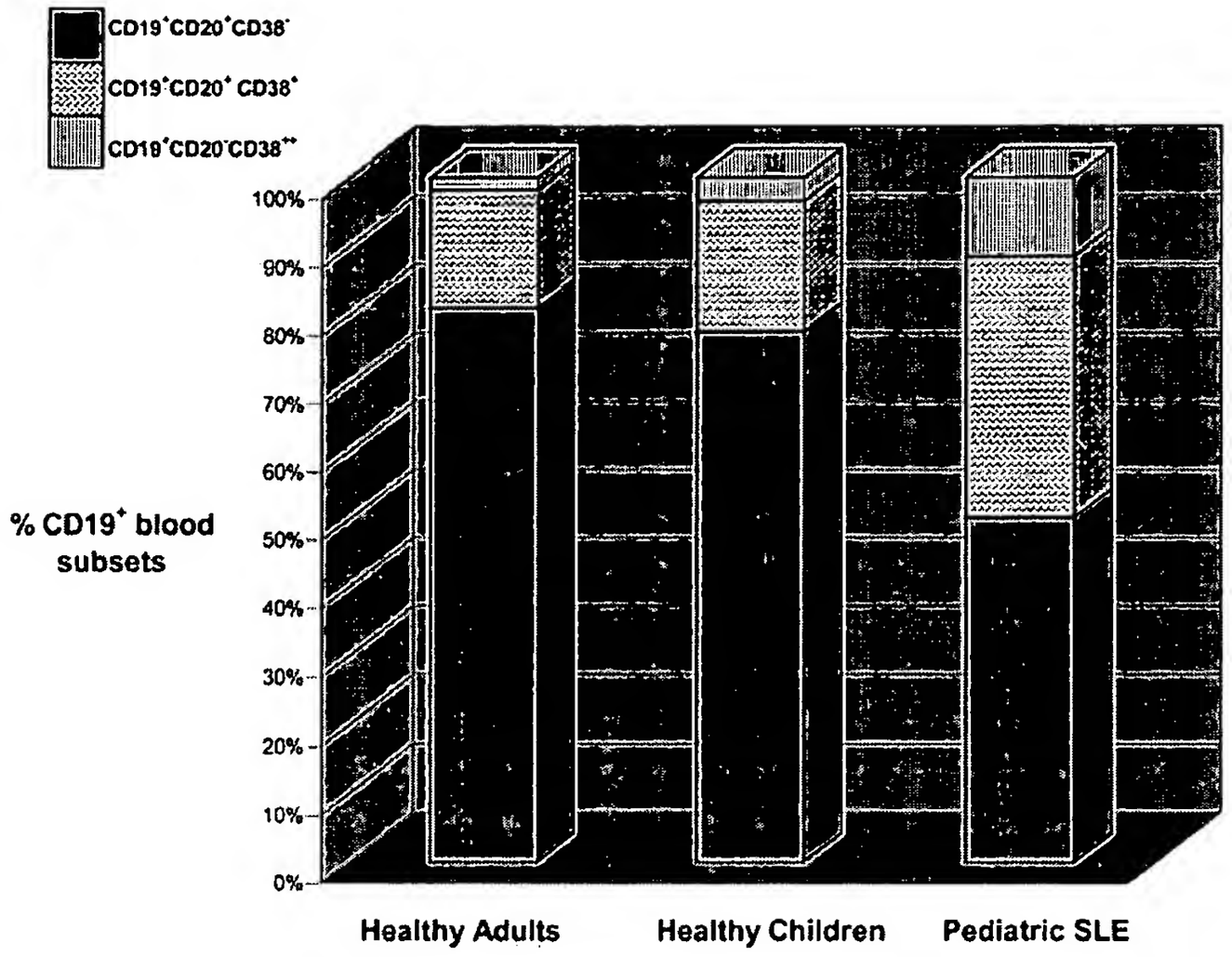

FIGURE 4. Percentage of conventional B cells ($CD19^+CD20^+CD38^-$), pre-GC B cells ($CD20^+$ $CD38^+$), and plasma cell precursors ($CD20^-CD19^{low}$ $CD27^{+/++}CD38^{++}$) in healthy adults, healthy children, and SLE patients.

similar to the tonsilar Bm2 ($IgD^+CD38^-CD23^+$) cell subset: they are larger than naive B cells and display a full cytoplasmic rim (Fig. 5, *a* and *b*). Using real-time PCR, these cells were found to transcribe centerin (Fig. 6), a GC-specific serpin not expressed in conventional naive and memory blood/tonsil B cells (36). Yet, the blood IgD^+CD38^+ cells seem less committed toward GC differentiation than the GC founder cells (Bm2′) that were previously identified within tonsils (37), as they mostly lack expression of CD10 and CD77, and only about one-fifth of these cells (21.5 ± 16.7% of 17 samples analyzed) express CD71.

One of the characteristics of tonsilar IgD^+CD38^+ cells is the initiation of somatic mutation within Ig V_H genes (38). Therefore, blood IgD^+CD38^+ cells were sorted from eight different SLE patients, and their V_H Ig RNA was amplified using primers specific to the small V_H4 and V_H5 family leader peptide and μ constant region. Fifty-six independent clones were sequenced and aligned to their closest germline counterparts, revealing the presence of low grade somatic mutation within 66% of the transcripts (1–7 bp substitutions/mutated V_H region; Table III). The same population in healthy adults showed a higher rate of mutation (80% transcripts), with a range of 1–13 bp

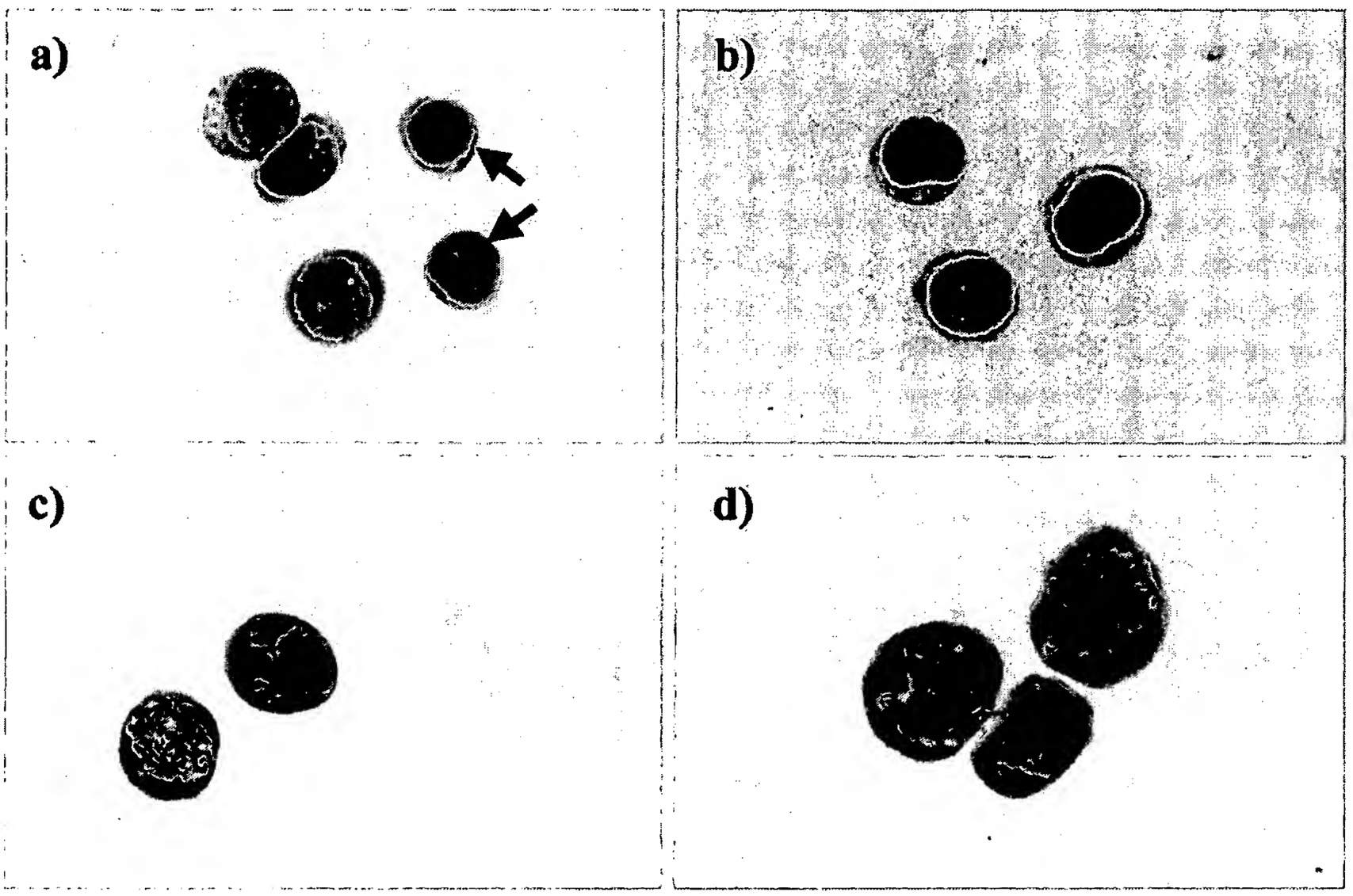

FIGURE 5. *a*, Wright-Giemsa staining of cytospun, magnetic bead-purified blood B cells; arrows show two resting naive B lymphocytes with scant cytoplasm next to three larger cells with more abundant cytoplasm corresponding to IgD^+CD38^+ B cells. *b*, Sorted blood IgD^+CD38^+ B cells. *c* and *d*, Sorted blood $CD19^{+/low}CD20^-CD27^+CD38^{++}$ plasmablasts at ×40 and ×100 magnifications, respectively.

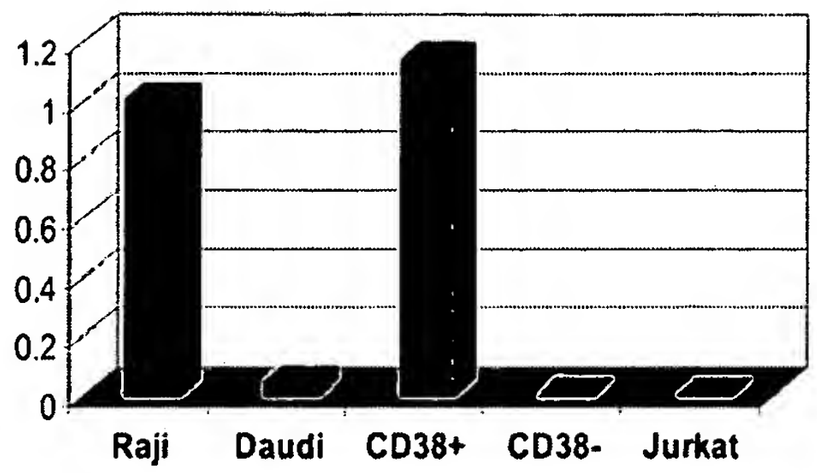

FIGURE 6. Expression of centerin message in the Burkitt's lines Raji and Daudi, IgD^+CD38^+ pre-GC B cells, IgD^+ and IgD^- $CD38^-$ (naive and memory) B cells, and the Jurkat T cell line. Quantification of centerin message was performed using real-time RT-PCR as described in *Materials and Methods*. Bars represent the relative expression of centerin in each of the tested samples using Raji RNA as standard. Values were normalized according to the ribosomal RNA expression in each of the samples.

substitutions/mutated V_H region (data not shown). Thus, blood IgD^+CD38^+ cells have initiated the process of somatic mutation. Taken together, our data indicate the presence in blood of a subset of B cells that may represent the link between naive and GC cells.

Increase in SLE blood of $CD20^-CD19^+CD38^{++}$ clonally expanded plasma cell precursors that can be further subdivided into $CD27^+$ and $CD27^{++}$

Most SLE patients display a distinct population of $CD20^-$ $CD19^{+/low}CD38^{++}$ blood cells (Fig. 7, *A* and *B*). Upon staining with CD27, these cells can be further subdivided into a $CD27^+$ and a $CD27^{++}$ population. Although the ratio of $CD27^+/CD27^{++}$ varies, the predominant population expresses CD27 with intensity comparable to that of memory ($CD27^+$) B cells (Fig. 7*B*). After sorting and Wright Giemsa staining, the majority of these cells do not look like mature plasma cells but like plasmablasts/early plasma cells (39, 40), as they have larger, less peripheral nuclei and less abundant cytoplasms (Fig. 5, *c* and *d*). The majority of these cells express both surface and intracytoplasmic Ig, with a $\kappa\lambda$ ratio close to 1 (43.5 ± 17.9% λ), whereas only a small percentage (15.5 ± 8.8%) of them expresses the mature plasma cell marker CD138 or syndecan.

As shown in Table II, SLE patients have a 3-fold expansion of this population compared with healthy controls. This expansion does not correlate with disease activity as measured by the SLEDAI (18.0 ± 19.9 cells/μl for SLEDAI <10; 24.1 ± 33.1 cells/μl for SLEDAI >10).

We sorted these cells and analyzed 38 IgG V_H gene transcripts from four different SLE patients. All but two transcripts showed a high frequency of somatic mutations (mean, 16 ± 8.5 mutations/mutated transcript). However, a striking finding was the identification in three of four patients of clonally related transcripts. An example of the V_H sequences corresponding to an expanded clone (seven related transcripts), with unique and shared mutations, is displayed in Fig. 8. The pattern of nucleotide mutation within this clone strongly suggests that it is the product of an Ag-driven response, as there is a high ratio of replacement vs silent substitution, especially concentrated within the second hypervariable region and the third framework. Clonally related, somatically mutated transcripts were also found in the blood plasma cell precursors isolated from two healthy adults (data not shown), suggesting that these cells in health and disease are the product of oligoclonal expansions.

SLE serum does not alter the survival of normal blood B cells

To determine whether the consistently low numbers of blood B cells and/or the activated B cell phenotype that we observed in our SLE patients were due to soluble serum factors, we purified naive blood and tonsilar B cells from healthy donors and cultured them in the presence of autologous sera, sera from four lymphopenic SLE patients with different SLEDAI, and sera from two patients with JDM. The percentage and absolute numbers of viable cells were calculated at 24, 48, 72, and 96 h using a hemocytometer after trypan blue staining. Apoptotic cells were also analyzed by flow cytometry using forward side scatter/side light scatter and annexin V binding/propidium iodine staining. No consistent differences were observed (data not shown), thus suggesting that a soluble factor(s) is not responsible for mature B cell death and subsequent lymphopenia in all SLE patients.

Discussion

B cell subsets in the blood of healthy children

Our study shows that blood B cells in all age groups include at least four subsets: 1) naive ($CD19^+CD20^+IgD^+CD38^-CD27^-$) B cells, 2) pre-GC ($CD19^+CD20^+IgD^+CD38^+CD27^-$) B cells, 3) memory ($CD19^+CD20^+CD38^-CD27^+$) B cells, and 4) plasma cell precursors ($CD19^+CD20^-CD27^{+/++}CD38^{++}$). When comparing children to adults, naive and memory B cells are 2.4-fold more abundant, whereas pre-GC B cells and plasma cell precursors are 3- and 4-fold expanded, respectively, in children.

A puzzling observation is the detection in blood of $sIgM^+$ $sIgD^+$ B cells bearing a phenotype similar to that of tonsil GC B cell founders. As GC B cells, these cells express CD38 and centerin, but, unlike GC founders (Bm2′) and centroblasts (Bm3), they lack the expression of CD10 and CD77. Furthermore, they are smaller than centroblasts, hence their denomination as pre-GC cells. Importantly, these cells have initiated the process of somatic mutation, which is another hallmark of GC reactions; sequencing the V_H Ig transcripts from sorted $sIgD^+sIgM^+CD38^+$ blood B cells from healthy adults revealed a mutation frequency similar to that described for tonsilar GC B cell founders (1–12 bp mutations/V_H region in 80% transcripts) and higher than that of naive B cells (1–2 bp mutations/V_H region in 50% transcripts) (33, 39). Thus, $IgM^+IgD^+CD38^+$ blood B cells may represent the link between naive (Bm1 and Bm2) and GC founders (Bm2′). It remains to be established whether these cells result from 1) activation in lymphoid sites and recirculation in the blood, or 2) activation in nonlymphoid sites followed by recirculation in the blood and later homing to peripheral lymphoid organs.

Table III. *Mutation analysis of V_H transcripts from SLE B cell subpopulations*

B Cell Subpopulation	No. Clones	Isotype	No. Mutated Clones	No. Mutations (Average/ST dev)	Mutation Range	Clonal Relatedness
IgD^+CD38^-	10	$\mu\delta$	0	—	—	No
IgD^+CD38^+	56	$\mu\delta$	37	1.4 ± 1.6	0–7	No
$CD19^+CD20^-CD38^{++}$	30	γ	28	15.6 ± 8.6	0–31	Yes

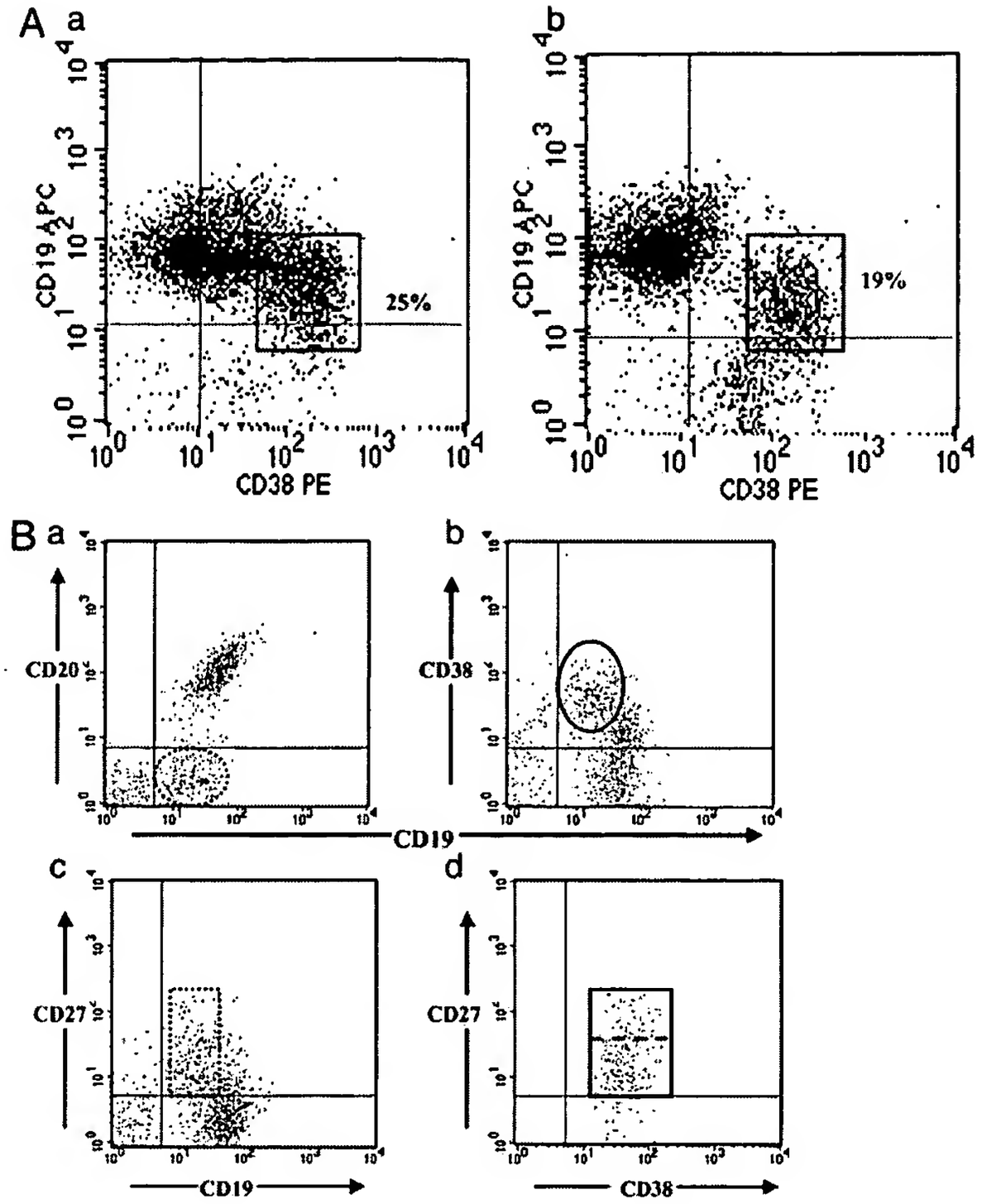

FIGURE 7. *A*, Enriched (>95% pure) blood B cells from two SLE patients (*A* and *B*). Squares depict the $CD19^{low}CD38^{++}$ plasma cell precursor population. Patient A was recently diagnosed and untreated at the time the sample was obtained, whereas the sample from patient B was obtained 1 mo after cytoxan and solumedrol pulses. *B*, Enriched B cells from a SLE patient stained with anti-CD20-PerCP, anti-CD19-allophycocyanin, anti-CD38-PE, and anti CD27-FITC. $CD19^{low}$ $CD38^{++}$ cells gated in *B* are represented within a rectangle in *D* and divided by a dotted line according to the intensity of CD27 staining. The same $CD19^{low}$ $CD38^{++}$ population is enclosed by a dotted circle (*A*) and a dotted rectangle (*C*). This experiment is representative of 12 individual experiments.

Plasma cell precursors constitute another underestimated circulating cell population. We show herein that they represent ~1.4% of the total B cell compartment in healthy adults and ~3.3% in healthy children. In the context of certain infections and malignancies, higher numbers of plasmablasts have been described in the blood (reactive plasmacytosis) (40, 41). These cells have been reported to characteristically lack the plasma cell marker CD138, but they acquire it in vitro upon exposure to IL-6 (41). Additionally, these cells express variable levels of CD27 (Refs. 42 and 43, and our own observation), suggesting caution when using CD27 to enumerate memory cells, especially in clinical situations where plasmacytosis may be expected.

Blood B cell subsets in children with SLE

Our studies reveal that children with SLE suffer profound B cell lymphopenia due to a dramatic reduction in all mature B cell subsets. SLE B cell lymphopenia does not correlate with any modality of therapy, SLEDAI, or anti-dsDNA or complement titers. SLE B cell lymphopenia could be due to 1) a reduction in the number of bone marrow B cell precursors, 2) shortened mature B cell life span, or 3) accelerated activation/differentiation of naive cells into downstream phenotypes including GC, memory, or plasma cells that would subsequently home into lymphoid tissues.

```
                                                                CDR1
VH5-251  MGSTAILALLLAVLQGVCA EVQLVQQSGAEVKKPGESLKISCKGSGYSF TSYWIG WVRQMP
SLE-1    ------------------- ----------------------------N- AGF--- ------
SLE-2    ------------------- ----------------------------N- AGF--- ------
SLE-3    ------------------- ----M------A-----------RA---T- A----A ----R-
SLE-4    ------------------- ------------------------------ AI---- ----V-
SLE-5    ------------------- -------------------T---E----K- A----- ------
SLE-6    ------------------- ----------------D-----------R- -T---- ----V-
SLE-7    ---------P--------- ------------------------------ AI---- ---HL-

                          CDR2                                          CDR3
VH5-251  GKGLEW MGIIYPGDSDTRYSPSFQGQ VTISADKSISTAYLQWSSLKASDTAMYYCAR SPGG
SLE-1    ------ -------------------- ---------N---------Q---------- SPGG
SLE-2    ------ ---------NA--------- I--------N---------R---------- ----
SLE-3    ------ ----F----EIT---P---- -I-----AMN--F---N--E---------- ----
SLE-4    ------ -T-------NA--------- I--------N---------Q---------- ----
SLE-5    ------ ---------NA--------- -I-----AMN--F---N--E---------- ----
SLE-6    --D--- ---------NA--------- I--------N---------Q---------- ----
SLE-7    ------ ---------NA--------- I--------N---------Q---------- ----
```

FIGURE 8. Amino acid translation of seven clonally related V_H5/γ transcripts isolated from sorted plasmablasts from the blood of a SLE patient. The transcripts display unique and common mutations while sharing the same V-D and D-J junctions (only the V-D junction is shown). There is a high ratio of R/S nucleotide substitutions especially within CDR2 (R/S = 2 and 5 in transcripts SLE 7 and SLE 3, the least and most mutated V_H regions from this clone, respectively) and FW3 (R/S = 3 and 8 in SLE 7 and SLE 3, respectively). The nucleotide sequences corresponding to these transcripts have been submitted to GenBank under accession numbers 384526, 384527, 384534, 384543, 384551, and 384565.

Killing of B cells by soluble factors (i.e., anti-lymphocytic Abs) has been implicated as a cause of SLE lymphopenia (44, 45). Although this mechanism may operate in some SLE patients, our results suggest that it is unlikely to explain the universal lymphopenia observed in this disease, as incubation of blood naive B cells from healthy donors with serum from active SLE patients failed to disclose any significant reduction in the number of viable cells. Additionally, the B and T lymphocyte propensity to undergo spontaneous and induced apoptosis has been recently described to be grossly intact in SLE (46).

The lymphopenia that we describe cannot be explained by bone marrow aplasia, as the neutrophil and platelet counts were within normal limits in the population that we studied. Furthermore, bone marrow aspirates from SLE patients, usually obtained in the context of severe blood cytopenias, have rarely revealed aplasias (47–49). Therefore, only a selective lymphoid cell precursor defect could explain the reduced numbers of T and B cells that we observed in the blood of our SLE patients. The increased proportion of $CD38^+$ B cells in SLE blood may provide us with some clues regarding the lymphopenia and perhaps some ethiopathogenic factors in this disease. In trying to induce naive B cells to become GC B cells in vitro, we identified IFN-α as one of the most efficient signals to up-regulate CD38 expression on naive B cells (50). Interestingly, high levels of IFN-α have been described in the serum of SLE patients (51), and the PBMCs of patients without circulating IFN-α display high levels of oligoadenylate synthetase and Mx protein, a signature of exposure to IFN-α (52, 53). The potential role of this cytokine in SLE development is further suggested by the large proportion of patients receiving IFN-α therapy who develop autoimmune, including SLE-like, syndromes (reviewed in Ref. 54). Finally, and perhaps best explaining the generalized lymphopenia of SLE patients, administration of IFN-α to newborn mice inhibits T and B cell development in the bone marrow, thymus, and spleen by 80% (55). Therefore, all these findings make it tempting to speculate that SLE may be associated with a deregulation of IFN-α production. Consistent with this hypothesis, the blood pre-GC (IgD^+CD38^+) B cell subpopulation is reduced to a lesser extent in SLE patients compared with controls and represents the predominant blood B cell population in many SLE patients.

In contrast to the reduction in all mature B cell subsets, children with SLE present a 3-fold expansion of blood plasma cell precursors that make up to 8.7% of their blood B cell compartment. Plasma cells expressing CD138 and high levels of CD27 have been recently reported in the blood of 13 adult SLE patients (43). In our study only a small proportion of the $CD20^-CD19^{low}CD38^{++}$ cells in the 68 patients analyzed display this more mature phenotype, whereas the majority lack CD138, express two levels of CD27 (comparable and higher than memory B cells), and upon sorting and Giemsa staining do not show a mature plasma cell morphology.

Blood plasma cell precursors are post-GC cells, as they express highly mutated and isotype-switched Ig transcripts. Additionally, there is a high degree of clonal relatedness within this subset, as numerous transcripts share the same VDJ rearrangement while displaying common and unique nucleotide substitutions. This suggests that they are the products of a recent clonal expansion that probably occurred in a GC, given the presence of unique mutations. This expansion may be explained by increased IL-10, a major plasma cell differentiation factor (56). Indeed, high levels of IL-10 are found in the serum of SLE patients, and treatment of these patients with anti-IL-10 Abs has shown beneficial effects (57–59). Alternatively, the recently identified B lymphocyte stimulator (BLyS/BAFF/TALL-1), a TNF family cytokine (60–63), may contribute to the disease, as it seems to prominently enhance humoral responses. BLyS transgenic mice show hypergammaglobulinemia and an autoimmune lupus-like disease (61). Furthermore, the survival of lupus-prone mice is increased by treatment with a BLyS antagonist (63). Although altered expression of BLyS and/or its receptors may play a role in human SLE, significant differences between the B cell phenotype found in BLyS transgenic mice and human SLE exist, as these transgenic mice display B cell expansion in the blood rather than the profound B cell lymphopenia that we describe in our patients. SLE may thus be best explained by the combined ectopic expression of cytokines such as α-IFN, IL-10, and BLyS. The etiology of this disease may be explained at the level of cells that produce these cytokines, which include APC such as dendritic cells.

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Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154-CD40 interactions

See the related Commentary beginning on page 1480.

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To determine the role of CD154-CD40 interactions in the B cell overactivity exhibited by patients with active systemic lupus erythematosus (SLE), $CD19^+$ peripheral B cells were examined before and after treatment with humanized anti-CD154 mAb (BG9588, 5c8). Before treatment, SLE patients manifested activated B cells that expressed CD154, CD69, CD38, CD5, and CD27. Cells expressing CD38, CD5, or CD27 disappeared from the periphery during treatment with anti-CD154 mAb, and cells expressing CD69 and CD154 disappeared from the periphery during the post-treatment period. Before treatment, active-SLE patients had circulating $CD38^{bright}$ Ig-secreting cells that were not found in normal individuals. Disappearance of this plasma cell subset during treatment was associated with decreases in anti–double-stranded DNA (anti-dsDNA) Ab levels, proteinuria, and SLE disease activity index. Consistent with this finding, peripheral B cells cultured in vitro spontaneously proliferated and secreted Ig in a manner that was inhibited by anti-CD154 mAb. Finally, the $CD38^{+/++}IgD^+$, $CD38^{+++}$, and $CD38^+IgD^-$ B cell subsets present in the peripheral blood also disappeared following treatment with humanized anti-CD154. Together, these results indicate that patients with active lupus nephritis exhibit abnormalities in the peripheral B cell compartment that are consistent with intensive germinal center activity, are driven via CD154-CD40 interactions, and may reflect or contribute to the propensity of these patients to produce autoantibodies.

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Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the production of multiple autoantibodies and by B cell hyperactivity that may either reflect intrinsic abnormalities or result from immunoregulatory defects in other cell types (1–4). Intrinsic or extrinsic perturbations of B cell maturation may permit generation, activation, differentiation, and clonal expansion of B cells that secrete pathogenic autoantibodies. Maturation of Ab responses occurs within germinal centers (GCs). Following activation in an antigen- and MHC-restricted manner, CD154-expressing T cells initiate the GC reaction by engaging CD40-expressing pre-switch IgD^+ or post-switch IgD^- B cells, thereby inducing them to express early-activation markers (CD69 and CD154) and differentiation markers (CD38, CD5, and CD27) (5–10) and to proliferate rapidly to form IgD^+ primary or IgD^- secondary follicles, more commonly referred to as GCs (11). Previous studies have defined functional B cell subsets from inflamed secondary lymphoid tissue, such as tonsil (12–21), or the periphery of active-SLE patients (22–29) by expression of CD27 and CD5, as well as IgD and CD38. Specifically, B cells that are bright for CD38, CD27, or CD5 have been shown to be Ig-secreting plasma cells, and cells expressing a low level of CD38, CD27, or CD5 have been shown to be memory-cell intermediates in the differentiation pathway to Ig-secreting plasma cells. Homotypic CD154-CD40 B cell interactions are essential for maintenance of ongoing GC reactions as well as for the differentiation of intermediates in the pathway to Ig-secreting plasma cells, such as from GCs to memory cells and from reactivated memory cells to Ig-secreting plasma cells (5, 30–38). The observation that T and B cells in

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Nonstandard abbreviations used: systemic lupus erythematosus (SLE); germinal center (GC); double-stranded DNA (dsDNA); phycoerythrin (PE); tricolor (TC; PE-Cychrome 5); intracytoplasmic (IC); mean fluorescence intensity (MFI); SLE disease activity index (SLEDAI).

the periphery of active-SLE patients spontaneously express CD154 suggests that GCs are abnormally releasing activated lymphocytes into the periphery, implying overactivity of GC reactions. Blocking CD154-CD40 interactions in vivo with humanized anti-CD154 (BG9588, 5c8) mAb in active-SLE patients may, therefore, interfere with the induction and maintenance of these ongoing GC reactions that produce autoantigen-specific memory and Ig-secreting plasma cells. The purpose of this study was to determine whether blocking CD154-CD40 interactions in vivo with a humanized mAb to CD154 (BG9588, 5c8) would interfere with the abnormal B cell activation in patients with active SLE and would also ameliorate signs and symptoms of the disease.

Methods

Patients and clinical data. The design of the clinical trial, including selection and exclusion of patients as well as preparation and administration of the humanized anti-CD154 mAb (BG9588, 5c8; Biogen Inc., Cambridge, Massachusetts, USA) and clinical monitoring, has been described (39, 40). Briefly, BG9588 consists of the complementarity-determining regions of the murine anti-human CD154 mAb 5c8, combined with human variable-region and framework-region residues and IgG1 and κ heavy and light chains, respectively. It was infused at a concentration of 20 mg/kg at weeks 0, 2, 4, 8, and 12 (Table 1). Patients receiving hydroxychloroquine were allowed to continue their therapy during the treatment period at a constant level (Table 1). Prednisone was tapered according to a predetermined schedule during the treatment period, beginning 1 month after the first dose of humanized anti-CD154 mAb (BG9588, 5c8) (40). The trial was prematurely terminated because of an increased frequency of thromboembolic events in patients in centers outside of the NIH. A total of six patients were treated at the Clinical Center of the NIH. This report describes four patients who received three or more infusions of humanized anti-CD154 mAb (BG9588, 5c8). All four female patients described in this article (age 34 ± 7 years, range 25–45 years) fulfilled the American College of Rheumatology criteria (41), had active lupus nephritis for 2.7–8 years before entry into the trial with mean proteinuria levels of 3.2 ± 1.4 g/24 h (range 1.7–4.9 g/24 h), and were positive for anti–double-stranded DNA (anti-dsDNA) Ab (188.5 ± 355.0 IU/ml, range 9.4–981.0 IU/ml, normal value <3.6 IU/ml). Active nephritis was defined as a renal biopsy showing proliferative lupus nephritis within 5 years before the first dose of humanized anti-CD154 mAb (BG9588, 5c8), proteinuria of at least 1.0 g/d at two separate screening visits, and any one of the following four criteria at each of the two screening visits: (a) anti-dsDNA Ab greater than twice the upper limit of normal (normal value <3.6 IU/ml); (b) complement protein 3 (C3) less than 80 mg/dl; (c) hematuria greater than 5 rbc's per high-power field; and (d) granular or rbc casts detected on urine analysis. Patient characteristics, concomitant treatment, and anti-CD154 schedules of treatment and assessment are summarized in Table 1.

Cell preparation and purification. PBMCs were obtained from non-autoimmune, healthy normal volunteers (n = 8) by centrifugation of collected heparinized blood over diatrizoate/Ficoll gradients (Sigma-Aldrich, St. Louis, Missouri, USA). PBMCs from the four active-SLE patients were obtained following collection of peripheral blood in vacutainer citrated cell-preparation tubes (Becton Dickinson, San Jose, California, USA) according to the manufacturer's instructions. Tonsillar mononuclear cells (n = 5–22) were obtained as described previously (5). Briefly, tonsils were minced and digested in RPMI containing 210 U/ml collagenase

Table 1
Patient characteristics

Patient	Ethnicity	Disease/ Nephritis duration (yr)	Clinical involvement	SLEDAI/ Anti-DNA (IU/ml)/Proteinuria (g/24 h)	Concomitant treatment	Anti-CD154 treatment schedule	Anti-CD154 assessment schedule
1 (45 yr, F)	Caucasian	8/8	Type IV GN, arthritis, malar rash, hematologic	12/23.9/4.85	Prednisone 25 mg qd	0, 2, 4, 8, 12 weeks	0, 2, 4, 8, 12, 16 weeks plus 3 and 20 months after final treatment
2 (31 yr, F)	Caucasian	9/7	Type III GN, arthritis, malar rash	8/31.6/1.67	Prednisone 5 mg qd, HCLQ 400 mg qd	0, 2, 4, 8 weeks	0, 2, 4, 8, 12 weeks plus 20 months after final treatment
3 (25 yr, F)	African-American	8/7.5	Type IV GN, arthritis, rash, pericarditis	8/9.4/4.42	Prednisone 20 mg qd, HCLQ 400 mg qd	0, 2, 4, 8 weeks	0, 2, 4, 8, 12 weeks plus 2 months after final treatment
4 (35 yr, F)	Asian	5/2.7	Type IV GN, arthritis, malar rash, pleuritis, subacute cutaneous LE, photosensitivity	18/981/1.85	Prednisone 30 mg qd, HCLQ 400 mg qd	0, 2, 4 weeks	0, 2, 4 weeks plus 20 months after final treatment

F, female; GN, glomerulonephritis; LE, lupus erythematosus; qd, four times a day; HCLQ, hydroxychloroquine.

type I (Worthington Biochemical Corp., Lakewood, New Jersey, USA) and 90 U/ml DNase (Sigma-Aldrich) for 30 minutes at 37°C. Following filtration through a wire mesh, the cells were washed twice in 20% FBS/RPMI and once in 10% FBS-RPMI before centrifugation over diatrizoate/Ficoll gradients.

In some cases, B cells were negatively selected on a magnetic column (StemCell Technologies, Vancouver, British Columbia, Canada) after staining with a mixture of dextran cross-linked mAb specific for glycophorin A, CD2, CD3, CD14, CD16, CD33, and CD56, followed by exposure to a magnetic colloid covalently linked to anti-dextran mAb. The resultant population of B cells was analyzed by flow cytometry and found to be more than 97% positive for sIg (FITC-conjugated anti-polyvalent Ig Ab; Caltag, Burlingame, California, USA). Alternatively, PBMCs were stained with phycoerythrin-conjugated (PE-conjugated) anti-CD19 (Becton Dickinson, San Jose, California, USA) and sorted for the $CD19^+$ population using the FACSVantage SE (Becton Dickinson).

Analysis of B cell function. B cells were cultured (1×10^5 per well) in U-bottom microtiter plates (Costar-Corning Inc., Corning, New York, USA) in RPMI (Life Technologies Inc., Grand Island, New York, USA) supplemented with penicillin G (200 U/ml), gentamicin (10 μg/ml), and 10% FCS. For analysis of the effects of CD154, cells were incubated in the presence of a previously described saturating concentration of 10 μg/ml (5) of the mouse anti-CD154 mAb (5c8; a kind gift from Biogen Inc.) or an isotype-matched control Ab (P1.17; mouse IgG2a; American Type Culture Collection, Manassas, Virginia, USA). In some cases, the humanized mouse anti-human CD154 mAb used in the clinical trial, or pooled human Ig (Sandoglobulin; Novartis, East Hanover, New Jersey, USA) as a control, was used.

To measure proliferation as assessed by DNA synthesis, cells were incubated in duplicate for various periods at 37°C with 1 μCi [^{3}H]TdR present for the last 18 hours of culture. Cells were harvested onto glass filter paper, and [^{3}H]TdR incorporation was determined by liquid scintillation spectroscopy.

IgM and IgG from pooled supernatants were analyzed by ELISA. Polystyrene microtiter plates were coated with capture Ab by incubation overnight at 37°C. After washing and blocking, coated plates were incubated with serial dilutions of standards or appropriately diluted culture supernatants for 16 hours at 37°C. Washed plates were incubated with peroxidase-conjugated goat anti-human IgM or IgG Ab for 1–2 hours at 37°C. After addition of substrate (*o*-phenylenediamine or chlorophenolred-β-D-galactopyranoside), the development of colored reaction product was quantitated using an ELISA reader.

Flow cytometric analysis. For analysis of activation-antigen expression, PBMCs were stained with FITC-conjugated anti-IgD (Caltag Laboratories Inc.), Tri-Color (TC; PE-Cychrome 5)-conjugated anti-CD19 (Caltag Laboratories Inc.), APC-conjugated anti-CD38 (Becton Dickinson), and PE-conjugated mAb against CD69 (Becton Dickinson) or CD154 (89-76; Becton Dickinson) as previously described (5). In some cases, CD154 was identified by staining with unconjugated anti-CD154 (5c8; Biogen Inc.) followed by PE-conjugated goat anti-mouse Ig as previously described (5). Alternatively, PBMCs were stained with a combination of FITC-conjugated anti-IgD, PE-conjugated anti-CD19 (Becton Dickinson), and TC-conjugated anti-CD5 (Caltag Laboratories Inc.); a combination of FITC-conjugated anti-CD27 (Becton Dickinson), PE-conjugated anti-IgD (Pharmingen), and TC-conjugated anti-CD19; or a combination of FITC-conjugated anti-IgD, PE-conjugated anti-IgM, and TC-conjugated anti-CD19. Cells were stained with mAb according to the manufacturer's instructions in PBS with 1% BSA at 4°C for 30 minutes. Cells were washed and resuspended in PBS with 1% BSA before analysis with FACSCalibur (Becton Dickinson). PAINT-A-GATE and CellQuest (Becton Dickinson) were used to analyze data generated by flow cytometry.

To determine the presence of Ig-secreting plasma cells and the cell cycle status of B cells, cells were fixed and permeabilized with FACSjuice or fixed with FACSlyse (Becton Dickinson) for 10 minutes in the dark at room temperature, washed with 1% BSA/PBS, and stained with FITC-conjugated goat $F(ab')_2$ anti-human Ig or IgD (Caltag) and APC-conjugated anti-CD38 mAb. Cells were incubated with hypotonic propidium iodide solution to identify the presence of apoptotic cells and cells in cycle. The percentage of cells that were positive for intracytoplasmic (IC) Ig and the mean fluorescence intensity (MFI) of IC Ig expression were determined using the CellQuest and PAINT-A-GATE programs (Becton Dickinson). Specifically, IC Ig expression was determined by subtraction of the histogram representing the combination of surface and IC Ig expression in permeabilized cells.

PCR analysis of CD154 mRNA. $CD19^+$ B cells were sorted from PBMCs stained with fluorochrome-conjugated anti-CD19 into 96-well PCR plates (Robbins Scientific Corp., Sunnyvale, California, USA) using a FACSVantage flow cytometer (Becton Dickinson). Total RNA was extracted using the RNeasy RNA isolation kit (QIAGEN Inc., Chatsworth, California, USA). From each patient, 0.1×10^6 sorted B cells were used for RNA preparation. Contaminating genomic DNA was removed using RQ1 RNase-free DNase (Promega Corp., Madison, Wisconsin, USA) according to the manufacturer's instructions. For conversion of mRNA into cDNA, SuperScript II Reverse Transcriptase (Life Technologies Inc.) was used according to the manufacturer's instructions. The following sequences of oligonucleotides were used as primers for the amplification of cDNA: CD154 sense, 5′-AGTCAGGCCGTTGCTAGTCAGT-3′; CD154 antisense, 5′-GGAAACAATGGAGACTGCAGGTA-3′; CD154 nested sense, 5′-AGTCAGGCCGTTGCTAGTCAGT-3′; CD154 nested antisense, 5′-TTATGAGGAGTGGGCAGGCTCAG-3′; CD40 sense, 5′-GCAGGCACAAACAAGACTGA-3′; CD40 anti-

sense, 5′-CGACTCTCTTTGCCATCCTC-3′; CD40 nested sense, 5′-GCCAAGAAGCCAACCAATAA-3′; CD40 nested antisense, 5′-CGACTCTCTTTGCCATCCTC-3′; β-actin sense, 5′-GTCCTCTCCCAAGTCCACACA-3′; β-actin antisense, 5′-TGGTCTCAAGTCAGTGTACAGGTAA-3′; β-actin nested sense, 5′-GTCCTCTCCCAAGTCCACACA-3′; β-actin nested antisense, 5′-CTCAAGTTGGGGGACAAAAAG-3′.

Statistics. Data are shown as the mean ± SEM and were tested for statistical significance using the Student's *t* test. Statistical significance in the figures and tables is indicated with pairs of symbols denoting specific comparisons.

Results

SLE B cells express functionally active CD154. Whereas T and B cells from the periphery of normal, non-autoimmune volunteers were largely unactivated and CD154-negative, CD154 was spontaneously expressed on the surface of B and T cells from the periphery of active-SLE patients at similar densities (Figure 1a). Notably, in a separately analyzed active-SLE patient who was not part of the humanized anti-CD154 mAb treatment study, CD154 spontaneously expressed on the surface of active-SLE B cells was equivalently identified by the 5c8 clone of anti-CD154 (Biogen Inc.) or a commercially available anti-CD154 mAb (89-76; Becton Dickinson) (Figure 1b). Whereas highly purified B cells from the periphery of normal, non-autoimmune volunteers did not proliferate or secrete Ig when cultured alone in vitro, cultured peripheral B cells from active-SLE patients spontaneously proliferated and secreted Ig in a manner that was inhibited significantly in the presence of a blocking anti-CD154 mAb (BG9588, 5c8) (Figure 1c).

Differences in expression of activation and differentiation antigens on B cells from the periphery of active-SLE patients compared with B cells from the periphery or secondary lymphoid tissues of normal non-autoimmune volunteers. A number of activation and differentiation markers were assessed to determine the functional status of the SLE B cells in greater detail (Table 2). B cells in the blood of the active-SLE patients were activated as assessed by bright expression of IgM and spontaneous expression of CD69 and CD154 (Table 2). A significantly lower percentage of B cells in the periphery of non-autoimmune normal volunteers spontaneously expressed these activation antigens. The percentages of active-SLE B cells expressing CD69 and CD154 as well as the densities of expression of CD69, CD154, and IgM were much higher on the pre-switch IgD^+ subset compared with the post-switch IgD^- subset. This trend was also noted for peripheral B cells from normal non-autoimmune volunteers.

The percentage of B cells expressing CD27 or CD5 and the level of expression was significantly higher in pre-switch IgD^+ cells from active-SLE patients compared with cells from the periphery or the secondary lymphoid tissue tonsil of non-autoimmune volunteers (Table 2). In addition, the level of CD27 or CD5 expressed on post-switch IgD^- B in the periphery of active-SLE patients was similar to that on cells from tonsil and was significantly higher than in the periphery of normal volunteers. Of interest, the percentage of post-switch IgD^- B cells from active-SLE patients that expressed CD5 was significantly greater than that of cells from the periphery or the secondary lymphoid tissue tonsil of non-autoimmune normal volunteers. Moreover, there was no significant difference in the percentage of post-switch IgD^- B cells expressing CD27 in the periphery of active-SLE patients compared with cells from the periphery or secondary lymphoid tissues of non-autoimmune volunteers.

The percentage of B cells expressing CD38 was significantly higher in pre-switch IgD^+ cells from active-SLE patients compared with cells from the periphery of normal non-autoimmune volunteers but was not significantly different from that in cells from the secondary lymphoid tissue tonsil (Table 2). In addition, the percentage of B cells expressing CD38 was significantly higher in pre-switch IgD^+ cells than in post-switch IgD^- cells. Examples of B cell subsets defined by expression of CD38 and IgD are shown in Figure 2a for one tonsil that contained all eight possible subsets ($n = 22$), two different normal non-autoimmune volunteers ($n = 8$), and one active-SLE patient who exhibited all eight possible subsets. Notably, comparison of these subsets revealed that, on average, the percentage of $CD38^{+++}IgD^+$ Ig-secreting plasma cells was significantly higher ($P = 0.035$) in the peripheral blood of the SLE patients (3.0% ± 1.1%, range 0–5%) than in the non-autoimmune normal volunteers (1.0% ± 0.5%, range 0–4%). Of interest, the percentage of $CD38^{+++}IgD^+$ plasma cells in the blood of active-SLE patients was comparable to the percentage found in the B cell compartment of chronically activated tonsil tissue (3.0% ± 1%, range 0–8%). Moreover, there was a significantly higher percentage of $CD38^+IgD^+$ B cells in the periphery of active-SLE patients (35.3% ± 4.5%, range 24–45%) than in tonsil tissues (17.0% ± 3.8%, range 1–33%; $P = 0.0096$), and there was a higher percentage of $CD38^{++}IgD^+$ B cells in the periphery of active-SLE patients (16.5% ± 6.1%, range 5–28%) than in both blood (4.5% ± 1.0%, range 0–8%; $P = 0.01$) and tonsil (3.0% ± 1.5%, range 1–14%; $P = 0.0089$) from normal non-autoimmune volunteers. Furthermore, there was no significant difference between the percentages of $CD38^-IgD^+$ B cells in the periphery of non-autoimmune controls (32.6% ± 9.5%, range 7.0–82.0%) and active-SLE patients, but there was a significantly higher percentage of $CD38^-IgD^+$ B cells in the periphery of active-SLE patients (21.8% ± 1.2%, range 20.0–25.0%) compared with the B cell compartment of tonsil tissue (6.0% ± 1.3%, range 1.0–13.0%; $P = 9.6 \times 10^{-6}$).

In contrast to the pre-switch IgD^+ peripheral B cell subsets as described above, there were no significant average differences in the post-switch IgD^- peripheral B cell subsets defined by CD38 in active-SLE

Table 2
Phenotypic analysis of human B cell subsets

	CD19⁺IgD⁺			CD19⁺IgD⁻		
	Normal PB (n = 8)	SLE PB (n = 4)	Tonsil (n = 5–22)	Normal PB	SLE PB	Tonsil
IgM						
%	77.7 ± 8.3^{x} (61–86)	95.5 ± 4.2^{u,x} (83–100)	65.8 ± 12.1^{f,u} (33–91)	17.7 ± 6.7^{k} (11–31)	92.3 ± 6.1^{b,k} (74–100)	23.0 ± 11.9^{f,b} (4–58)
MFI	60.0 ± 12.3^{a} (37–79)	657.8 ± 173.6^{a,q} (157–952)	136.0 ± 38.7^{q} (47–236)	21.7 ± 5.2^{g} (15–32)	116.5 ± 36.1 (26–195)	92.0 ± 26.6^{g} (35–162)
CD69						
%	37.8 ± 11.4$^{\chi}$ (3–92)	70.5 ± 12.0$^{\chi}$ (47–99)	54.6 ± 7.9 (28–75)	20.0 ± 11.5 (2–99)	38.5 ± 11.9 (8–66)	37.4 ± 7.5 (17–61)
MFI	172.6 ± 57.1$^{\delta}$ (42–421)	305.8 ± 116.8^{g} (84–579)	58.8 ± 13.9^{g} (7–90)	38.3 ± 3.1$^{h,\delta,\phi}$ (28–52)	78.3 ± 27.1$^{\phi}$ (33–150)	65.6 ± 3.0^{h} (56–72)
CD154						
%	20.4 ± 5.3$^{\varphi}$ (4–44)	77.3 ± 12.3$^{\varphi,\pi,r}$ (55–99)	16.5 ± 6.9^{r} (3–49)	6.1 ± 3.8 (0–31)	17.0 ± 7.5$^{\pi}$ (2–36)	9.4 ± 5.3 (0.4–37)
MFI	930.8 ± 281.3$^{\lambda,v}$ (175–2,521)	1,054.3 ± 244.4^{s} (559–1,553)	284.5 ± 167.0^{s,v} (33–1,432)	236.8 ± 88.3$^{\lambda,w}$ (0–705)	1,264.3 ± 517.6^{t} (169–2,969)	53.5 ± 17.4^{w,t} (20–138)
CD27						
%	36.2 ± 6.9^{9,13} (19–62)	97.0 ± 2.1^{9,m} (93–100)	38.4 ± 7.5^{j,m} (24–62)	66.7 ± 7.7^{13} (49–89)	72.7 ± 9.9 (59–92)	58.4 ± 4.0^{j} (45–67)
MFI	75.2 ± 17.8^{11} (29–142)	467.3 ± 157.0^{11,n} (164–689)	87.2 ± 14.8^{n} (39–130)	39.8 ± 6.0^{12,o} (19–60)	117.0 ± 41.0^{12} (39–178)	76.0 ± 19.1^{o} (22–135)
CD5						
%	51.3 ± 8.8^{2} (27–93)	87.5 ± 8.4^{2,c} (64–100)	45.1 ± 13.3^{c} (5–98)	43.3 ± 12.6^{3} (6–94)	85.0 ± 11.6^{3,e} (51–100)	36.4 ± 15.6^{e} (1–96)
MFI	528.3 ± 130.3^{4,6} (71–1,114)	1,247.8 ± 439.3^{4,7,d} (371–2,464)	276.3 ± 102.7^{d} (48–811)	85.7 ± 11.0^{5,6} (32–112)	162.8 ± 49.4^{5,7} (94–306)	200.9 ± 95.2 (29–760)
CD38						
%	49.1 ± 11.0 (5–88)	71.0 ± 5.3$^{\beta}$ (57–81)	73.6 ± 4.0 (5–90)	45.0 ± 9.6 (4–72)	51.8 ± 3.7$^{\beta}$ (45–62)	70.1 ± 5.0 (1–92)
MFI	139.0 ± 25.0$^{\alpha}$ (42–208)	412.5 ± 200.2$^{\alpha}$ (187–1,012)	259.7 ± 84.8^{p} (39–2,021)	493.1 ± 225.0 (49–1,640)	442.0 ± 268.4 (125–1,245)	462.2 ± 71.1^{p} (30–1,739)

Statistical significance (P < 0.05; range 0.00003–0.049) is indicated with pairs of symbols denoting specific comparisons. Ranges are shown in parentheses. PB, peripheral blood.

patients compared with normal peripheral B cells. When compared with tonsil, the periphery of both active-SLE patients and normal non-autoimmune healthy volunteers had fewer CD38⁺IgD⁻ and CD38⁺⁺IgD⁻ B cells.

Expression of differentiation and activation antigens during and after treatment of active-SLE patients with humanized anti-CD154 mAb (BG9588, 5c8). CD38^{positive} B cells in the circulation of the active-SLE patients disappeared from the peripheral blood during the treatment regimen with humanized anti-CD154 mAb (Figures 2b, 2c, and 3a). Specifically, before the treatment regimen, 65% ± 5.5% of the B cells were CD38^{positive}. At 4–8 weeks after initiation of treatment, the percentage of CD38^{positive} B cells in the circulation had dropped to 7.0% ± 4.1% (P = 0.00017 compared with before treatment). Withdrawal of treatment led to a reappearance of CD38^{positive} B cells in the circulation (73.8% ± 6.9%; P = 0.00018) at the earliest time point tested, 2 months after treatment, at a percentage that was not different from the pretreatment percentage (P > 0.05). Of interest, this trend was significant for both the pre-switch IgD⁺ and the post-switch IgD⁻ B cell subsets.

Figures 2b and 2c depict the effect of humanized anti-CD154 mAb treatment on the individual peripheral B cell subsets in active-SLE patients, defined by CD38 and IgD. Four to eight weeks of treatment led to a significant reduction in the peripheral CD38⁺/⁺⁺IgD⁺ B cell and CD38⁺⁺⁺IgD⁺ plasma cell populations and a significant mean increase in the CD38⁻IgD⁻ peripheral B cell population. After treatment was discontinued, these effects dissipated. Of note, the CD38⁺⁺⁺IgD⁻ plasma cells that were present in the periphery of SLE patient no. 3 (SLE no. 3) disappeared from the periphery following two treatments with humanized anti-CD154. Interestingly, the CD38⁺⁺⁺ cells that reappeared in the circulation of SLE no. 3 two months after withdrawal of treatment were pre-switch IgD⁺ plasma cells (data not shown).

By contrast, the percentage of CD69$^+$ B cells observed in the periphery of SLE patients (57.0% ± 9.0%, range 13.0–100.0%; MFI 269.3 ± 168.5, range 59–633) did not significantly change during the treatment period (91.9% ± 4.7%, range 81–100%; MFI 171 ± 39.6, range 101–279) but did decrease after the treatment was withdrawn (62.3% ± 7.3%, range 46–79%; MFI 305.8 ± 134.7, range 59–633; $P = 0.011$) (Figure 3b). Separate analysis of the pre-switch IgD$^+$ and post-switch IgD$^-$ subsets revealed different trends of CD69 expression in response to treatment. Specifically, the pre-switch IgD$^+$ subset followed the trend observed in the whole B cell population. By contrast, the percentage of CD69$^+$ cells in the post-switch IgD$^-$ subset significantly increased following 4–8 weeks of treatment and then significantly decreased during the post-treatment period.

Similar to the disappearance of CD69$^+$ B cells from the circulation following withdrawal of anti-CD154 treatment, the CD154$^+$ pre-switch IgD$^+$ B cells observed in the periphery of SLE patients decreased after the treatment was stopped (Figure 4). Of note, CD154 protein expression on the surface of cells could not be examined during the time that anti-CD154 mAb was being administered to the patients, since the treatment itself covered up available CD154 epitopes. For this reason, PCR analysis was used to monitor expression of CD154 mRNA in individually sorted CD19$^+$ B cells at the pretreatment time point and after initiation of treatment. These experiments demonstrated that expression of CD154, and, as controls, β-actin and CD40, had not changed at 1 month after treatment initiation.

Figure 5 depicts the effect of humanized anti-CD154 mAb treatment on the expression of CD27 on circulating peripheral B cells of individual active-SLE patients. Histograms of CD27 expression in normal non-autoimmune volunteers are shown for comparison. The percentage of pre-switch IgD$^+$ B cells expressing CD27 significantly decreased following treatment with anti-CD154 (pretreatment, 98.0% ± 1.2%, range 95–100%; during treatment, 73.0% ± 1.9%, range 69–77%; post-treatment, 43.0% ± 22.2%, range 13–97%; $P = 0.0004$). There were no significant changes in CD27-expressing B cells in the post-switch IgD$^-$ subset. Individual patients exhibited different trends. For example, the percentage and MFI of CD27-expressing pre-switch IgD$^+$ cells in SLE nos. 1 and 2 continued to remain low in the post-treatment time period, whereas CD27-positive pre-switch IgD$^+$ cells reappeared in the periphery of SLE no. 3 following treatment withdrawal. These trends are reflected in the finding that, on average, pre-switch IgD$^+$ cells expressing a low level of CD27 (CD27$^{low/+}$) were significantly reduced ($P = 0.0003$) when comparing this population during treatment

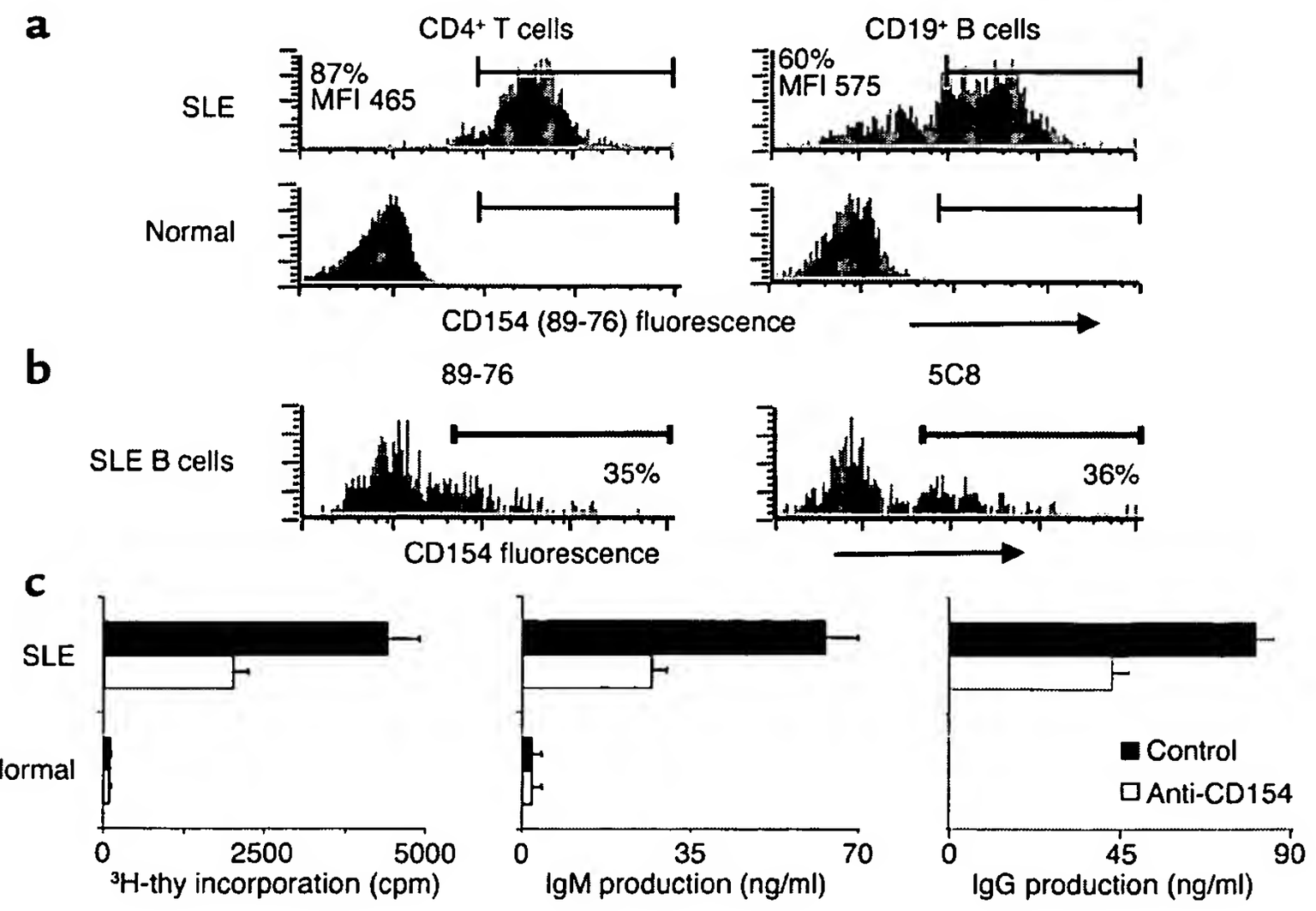

Figure 1

Freshly isolated SLE B cells express functionally active CD154. (**a** and **b**) CD154 expression on CD4$^+$ T cells and CD19$^+$ B cells from the periphery of SLE patients and normal volunteers was assessed by flow cytometric staining of PBMCs for CD154 (PE-conjugated 89-76; shown in **a** and in **b** [left panel]; unconjugated 5c8 followed by PE-conjugated goat anti-mouse Ig, **b** [right panel]) and CD19 or CD4 (APC-conjugated mAb, **a** and **b**). The results of two of six experiments with similar findings are shown. (**c**) Freshly isolated, negatively selected peripheral B cells (0.1×10^5) from active-SLE patients and normal volunteers were incubated in the presence of 10 μg/ml anti-CD154 (mIgG2a; 5c8) or 10 μg/ml control Ab (mouse IgG2a; P1.17). Analysis of DNA synthesis was carried out on day 3. Ig production was analyzed by ELISA after a 5-day incubation. All determinations were performed in duplicate and are expressed as the mean ± SEM. The results of one of three experiments with similar findings are shown. ^{3}H-thy, ^{3}H-thymidine.

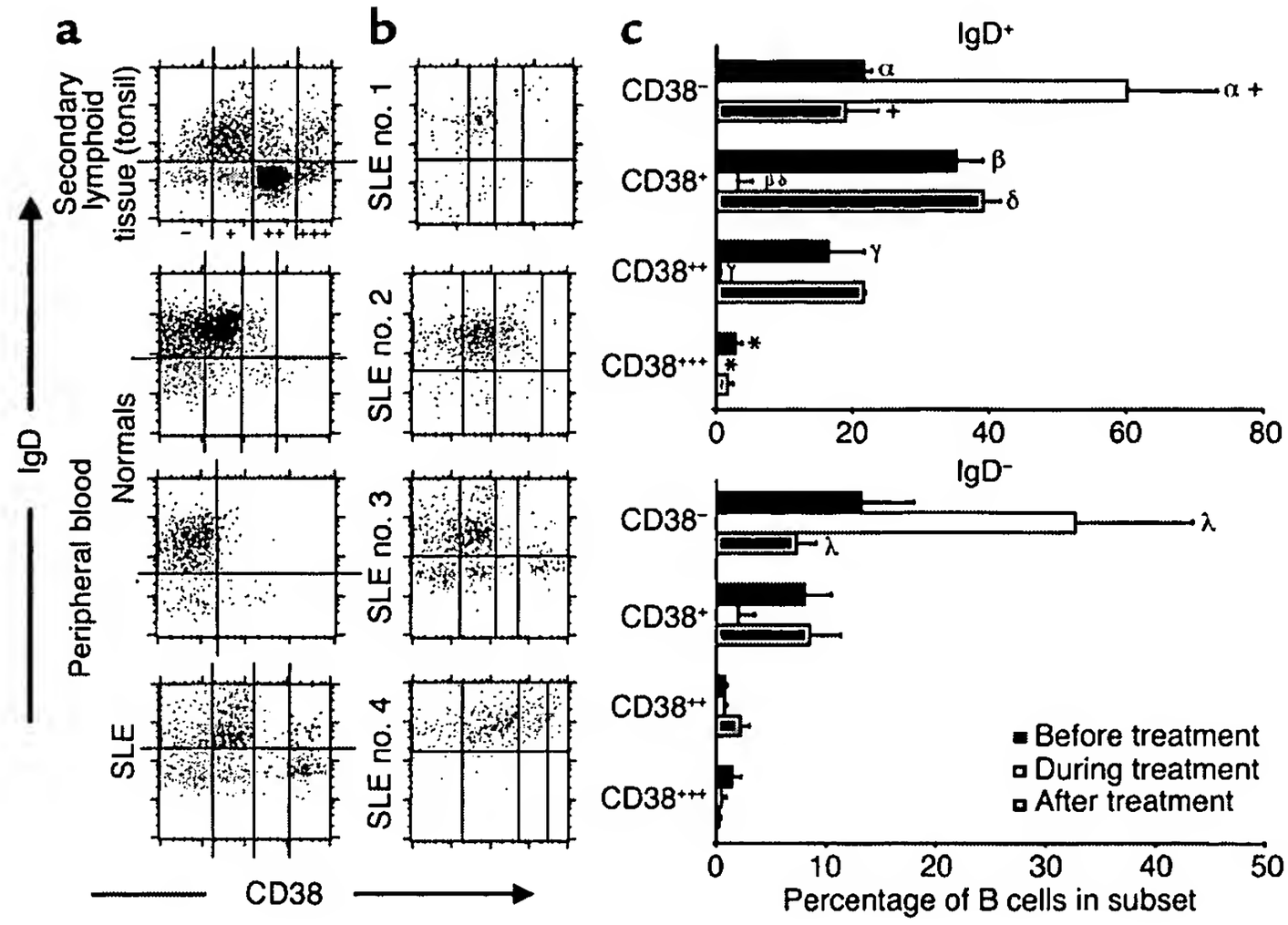

Figure 2

CD38-expressing B cell subsets in active-SLE blood disappear following two treatments with humanized anti-CD154 (5c8). Freshly isolated mononuclear cells (MNCs) from active-SLE patients (**a** and **b**), normal volunteers (**a**), and individual tonsils (**a**) were assessed for $CD19^+$ subpopulations by FACS analysis following staining with FITC-conjugated anti-IgD, PE-conjugated anti-CD38, and APC-conjugated anti-CD19. Freshly isolated PBMCs from active-SLE patients before treatment, during treatment (SLE nos. 1 and 2, 2 months; SLE nos. 3 and 4, 1 month), and after treatment withdrawal (SLE no. 1, 3 months after; SLE no. 2, 20 months after; SLE no. 3, 2 months after; SLE no. 4, 20 months after) were assessed for $CD19^+$ subpopulations by FACS analysis following staining with FITC-conjugated anti-IgD, PE-conjugated anti-CD38, and APC-conjugated anti-CD19 (**c**). The mean ± SEM percentages of $CD19^+$ B cells in each subset defined by CD38 and IgD are shown graphically. Statistical significance was determined by the Student's *t* test and is depicted with each pair of symbols indicating a specific comparison: $^{\lambda}P = 0.0445$, $^{\alpha}P = 0.0208$, $^{+}P = 0.0208$, $^{\beta}P = 0.00037$, $^{\delta}P = 0.014$, $^{\gamma}P = 0.0197$, $^{*}P = 0.016$.

with the post-treatment time point (pretreatment, 38.3% ± 18.4%, range 13–74%; during treatment, 50.3% ± 5.2%, range 40–56%; post-treatment, 22.3% ± 11.9%, range 8–46%). In addition, $CD27^-IgD^+$ cells were significantly increased (pretreatment, 2.3% ± 1.5%, range 0–5; during treatment, 27.3% ± 2.3%, range 23–31%; post-treatment, 57.3% ± 27.2%, range 3–87%) ($P = 0.04$) when comparing this population during treatment with the post-treatment time point.

Figure 6 depicts the effect of humanized anti-CD154 mAb treatment on the expression of CD5 on circulating peripheral B cells of active-SLE patients. Although no significant average differences were observed between the pre-switch IgD^+ and the post-switch IgD^- B cell subsets in the percentage or density of total CD5 expression following treatment, there were significant differences within individual subsets. Notably, the pre-switch cells that were bright for CD5 ($CD5^{+++}IgD^+$) significantly decreased ($P = 0.02$) in the periphery following 4–8 weeks of humanized anti-CD154 treatment (pretreatment, 18.3% ± 3.4%, range 14–25%; during treatment, 7.7% ± 1.8%, range 5–11%; post-treatment, 8.7% ± 3.8%, range 3–16%). In addition, both pre-switch IgD^+ and post-switch IgD^- B cells expressing a low level of CD5 ($CD5^+$) followed the same trend after humanized anti-CD154 treatment with respect to expression of CD69 and CD154. On average, the $CD5^+$ B cell subset did not change during the treatment period but significantly decreased ($P < 0.04$) following treatment withdrawal (IgD^+: pretreatment, 46.3% ± 10.7%, range 46–81%; during treatment, 49.7% ± 10.8%, range 28–61%; post-treatment, 11.7% ± 3.3%, range 5–16%; IgD^-: pretreatment, 65.6% ± 10.3%, range 46–81%; during treatment, 62.0% ± 12.1%, range 38–76%; post-treatment, 25.3% ± 9.5%, range 13–44%). Different trends were noted in SLE nos. 2 and 3. Specifically, in SLE no. 2, the pre-switch IgD^+ and post-switch IgD^- subsets that were positive for CD5 decreased in both percentage and MFI during treatment and after treatment withdrawal so that in the post-treatment period the pre-switch cells were mostly CD5-negative. In SLE no. 3, treatment with humanized anti-CD154 mAb decreased the density of CD5 on the surface of both pre-switch IgD^+ and post-switch IgD^- B cells; this decrease was reversed in the post-treatment time period.

Treatment with humanized anti-CD154 mAb (BG9588, 5c8) has no effect on circulating lymphocyte numbers. Blocking CD154-CD40 interactions with humanized anti-CD154 mAb (BG9588, 5c8) did not significantly affect the total number of mononuclear cells, $CD4^+$ T cells, or $CD19^+$ B cells found in the peripheral

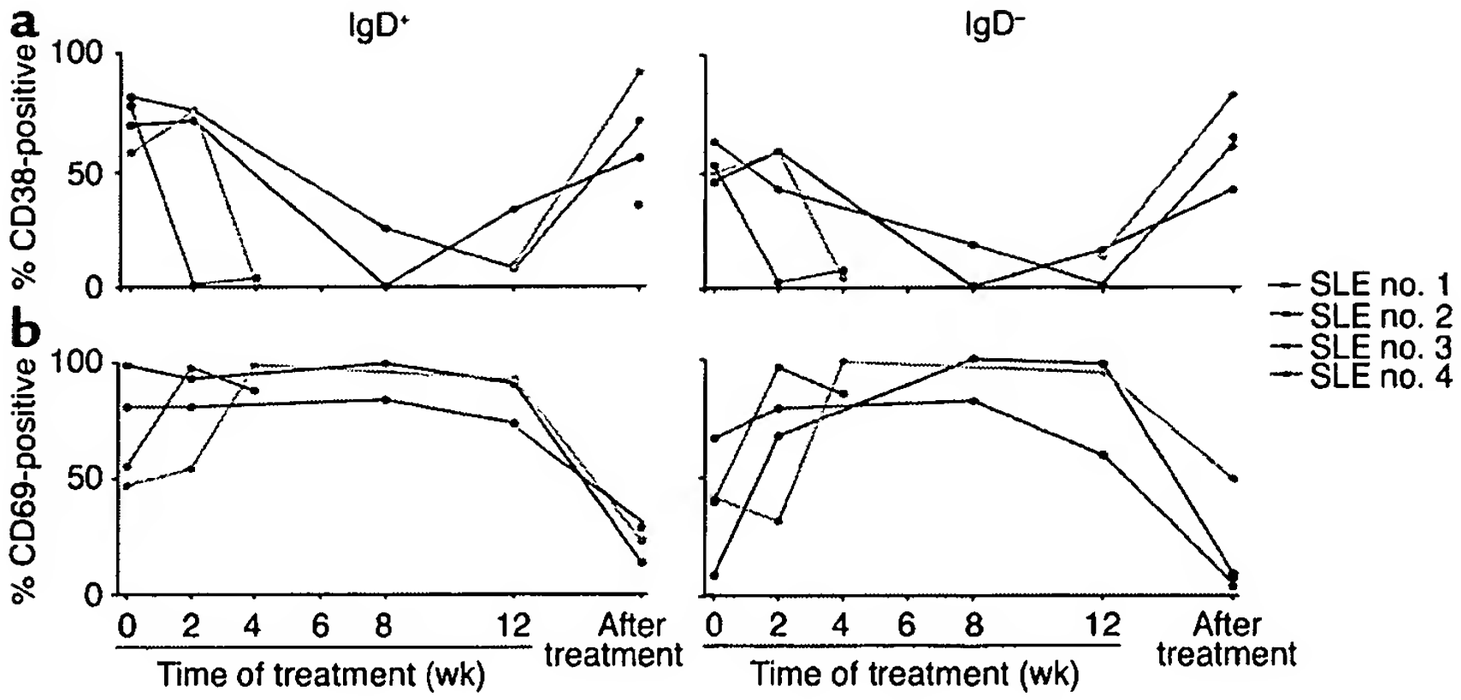

Figure 3
Expression of activation antigens by B cells diminishes with different kinetics following treatment of active-SLE patients with humanized anti-CD154 (5c8). Freshly isolated PBMCs from normal volunteers and active-SLE patients before treatment, during treatment, and after treatment (SLE no. 1, 2 months after; SLE no. 2, 20 months after; SLE no. 3, 3 months after; SLE no. 4, 20 months after) were assessed for CD69 and CD38 expression in $CD19^+$ B cell subsets by FACS analysis following staining with FITC-conjugated anti-IgD, APC-conjugated anti-CD19, and PE-conjugated Ab against CD69 or CD38. The percentages of $CD19^+$ pre-switch IgD^+ and post-switch IgD^- B cells expressing CD38 (**a**) and CD69 (**b**) before, during, and after treatment are shown.

blood of these patients (40). Moreover, treatment of active SLE nephritis patients with humanized anti-CD154 did not significantly change the ratio of pre-switch IgD^+ to post-switch IgD^- B cells in the periphery (before treatment, 6 ± 3, range 1–12; 2 weeks after treatment initiation, 5 ± 3, range 1–13; 1 month after treatment initiation, 3 ± 2, range 1–3; 2 months after treatment initiation, 5 ± 3, range 1–6; after final treatment, 2 ± 1, range 1–4; $P > 0.05$).

Spontaneously proliferating B cells and Ig-secreting B cells disappear from the peripheral blood of active-SLE patients following treatment with humanized anti-CD154 mAb (BG9588, 5c8). A hallmark of active SLE is the presence of spontaneously proliferating B cells in the periphery (Figure 1c). For SLE patient nos. 1 and 2 who participated in the anti-CD154 mAb trial, spontaneously proliferating B cells were observed in the periphery as assessed by the percentage of peripheral B cells in the $S/G_2/M$ stages of the cell cycle (Figure 7a). Two treatments with humanized anti-CD154 mAb decreased the presence of these cells in the circulation, but they reappeared in the circulation following withdrawal of treatment. Of note, B cells in the periphery of non-autoimmune normal volunteers do not spontaneously proliferate (Figure 1c) and thus are in the G_0 or G_1 stage of the cell cycle (data not shown).

Circulating Ig-secreting cells were identified in the blood of two of four of the SLE patients (nos. 1 and 3). Whereas less than 1% of the PBMCs isolated from SLE no. 1 were positive for IC Ig (data not shown), 3% of the PBMCs isolated from SLE no. 3 were positive for IC Ig (Figure 7b). As expected, all of these cells were $CD38^{bright(+++)}$. Interestingly, 60% of the IC Ig^+ cells were in the S, G_2, or M phase of the cell cycle, indicating that they were dividing plasma cells, whereas the remaining 40% were in the G_0 or G_1 phase, representing nonproliferating plasma cells. Treatment of SLE no. 3 with humanized anti-CD154 mAb resulted in complete and

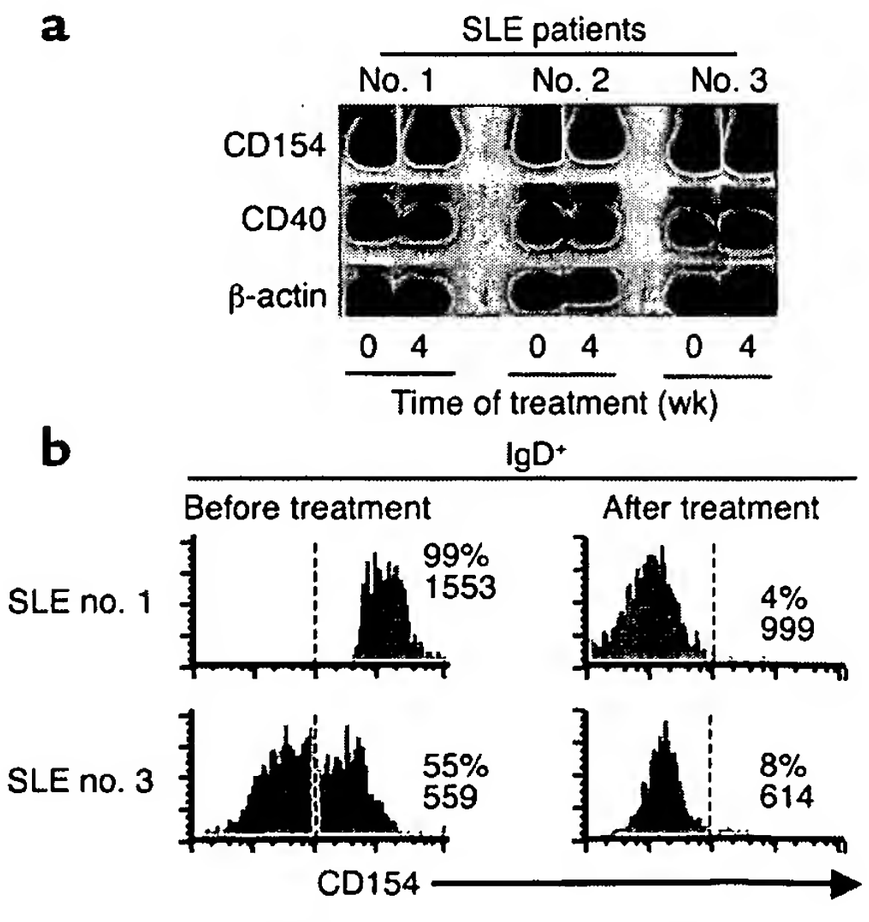

Figure 4
CD154 expression by active-SLE B cells after a treatment course with humanized anti-CD154 (5c8). Individual $CD19^+$ B cells in freshly isolated PBMCs from normal volunteers and active-SLE patients before treatment, after two treatments, and 2–3 months after treatment were identified following staining with fluorochrome-conjugated anti-CD19 and either sorted into individual wells using a FACSVantage flow cytometer (**a**) or additionally stained with PE-conjugated anti-CD154 (89-76; **b**). Transcripts of CD154, CD40, and β-actin mRNA were identified by PCR and Southern blotting (**a**). Surface expression of CD154 is shown in **b**, where the dotted line indicates the cutoff for staining with a PE-conjugated isotype-matched control Ab.

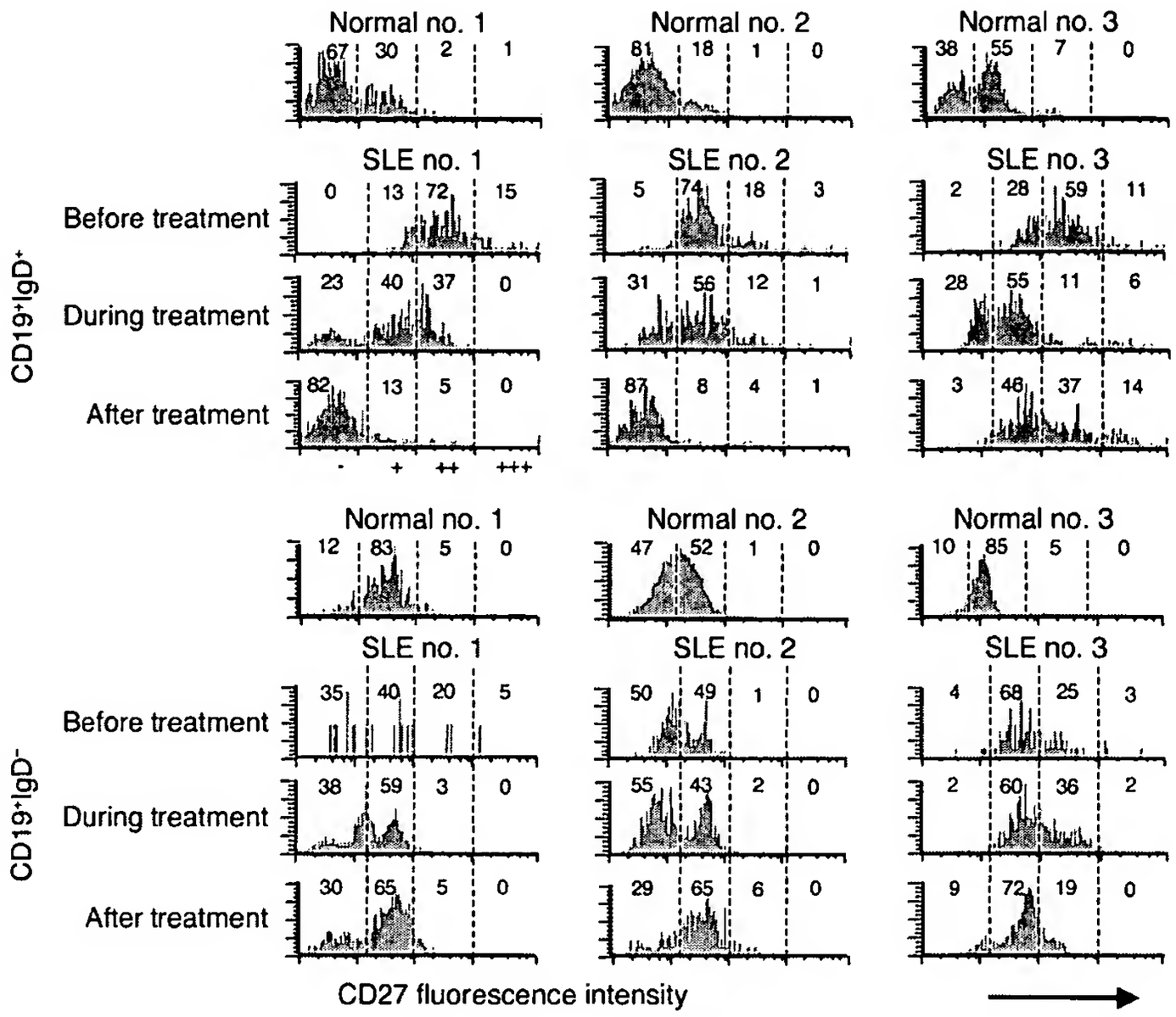

Figure 5
Impact of treatment with humanized anti-CD154 Ab (5c8) on CD27-expressing B cell subsets. Freshly isolated PBMCs from active-SLE patients and normal volunteers were assessed for CD27 expression in B cell subsets by FACS analysis following staining with FITC-conjugated anti-CD27, PE-conjugated anti-IgD, and TC-conjugated anti-CD19. Histograms of CD27 expression on B cells from normal volunteers or active-SLE patients before treatment, during treatment (SLE nos. 1 and 2, 2 months; SLE no. 3, 1 month), or after withdrawal of treatment (SLE no. 1, 3 months after; SLE no. 2, 20 months after; SLE no. 3, 2 months after) are shown.

sustained disappearance of Ig-secreting cells from the peripheral blood for the duration of the treatment regimen. Two months after withdrawal of treatment with humanized anti-CD154 mAb, the Ig-secreting cells reappeared in the peripheral blood (Figure 7b).

Treatment of active-SLE patients with humanized anti-CD154 mAb (BG9588, 5c8) decreases anti-dsDNA Ab levels, decreases the degree of proteinuria, and improves the SLE disease activity index score. The overall clinical response observed in the multicenter trial has been reported (40). All four patients included in this analysis showed some improvement during and after the treatment period with humanized anti-CD154 mAb (BG9588, 5c8) (Figure 8). A treatment-related decrease in anti-dsDNA Ab was seen in all patients. The pretreatment level of serum anti-dsDNA Ab was 188.5 ± 355 IU/ml (100%; range 9.4–981 IU/ml, normal value <3.6 IU/ml). After two treatments, serum anti-dsDNA significantly decreased to 64% ± 19% of the pretreatment value (range 32–81%; $P = 0.008$). In the three patients who received more than three treatments, a significant decrease in serum anti-dsDNA Ab was observed after three and four treatments (2 and 3 months), to 66% ± 2% (range 63–68%; $P = 0.001$) and 63% ± 7% (range 54–69%; $P = 0.0001$) of the pretreatment value, respectively. Six months after treatment was withdrawn, serum anti-dsDNA Ab rebounded to 113% ± 33% (range 67–157%) of the pretreatment level. Gradual increases started at 2 months after treatment, to 61% ± 30% (range 26-105%) of pretreatment levels, and continued 3 months after treatment to 80% ± 25% (range 60–122%) of pretreatment levels.

The mean proteinuria for all four patients enrolled in the anti-CD154 trial was 3.6 ± 1.4 g/24 h (100%; normal value 0 g/24 h) at the pretreatment time point. Following two treatments, proteinuria was 94% ± 9% of the pretreatment level (3.4 ± 1.5 g/24 h; $P > 0.05$). Proteinuria was significantly decreased from the pretreatment value following three treatments at the 2-month time point (2.5 ± 1.8 g/24 h, 68% ± 35% of the pretreatment level; $P < 0.05$). This significant decrease was sustained at the 3-month time point (four treatments, 2.1 ± 1.1 g/24 h, 60% ± 21% of the pretreatment level) and in the post-treatment period (3 months, 1.9 ± 1.5 g/24 h, 65% ± 31% of the pretreatment level; 6 months, 1.6 ± 1.9 g/24 h, 48% ± 41% of the pretreatment level; $P < 0.05$). A long-term effect of anti-CD154 treatment on kidney function was suggested by the finding that at the 20-month post-treatment time point, mean proteinuria was 0.6 ± 0.6 g/24 h (32% ± 31% of the pretreatment level) in three of four patients with a prolonged response.

SLE disease activity index (SLEDAI) was measured at a pretreatment time point, during the course of treatment, and after treatment was withdrawn. The mean SLEDAI for all four patients enrolled in the anti-CD154 trial was 10.6 ± 3.9 (100%) at the pretreatment time point, 7.3 ± 4.7 (71% ± 42%) after two treatments, and 4.0 ± 1.6 (42% ± 12% of the pretreatment score; $P < 0.05$) at the 2-month time point after three treatments. This favorable effect of anti-CD154 treatment on SLEDAI was persistent after cessation of treatment, as the mean SLEDAI recorded 3 months after the final treatment was 5.6 ± 3.7 (52% ± 30% of

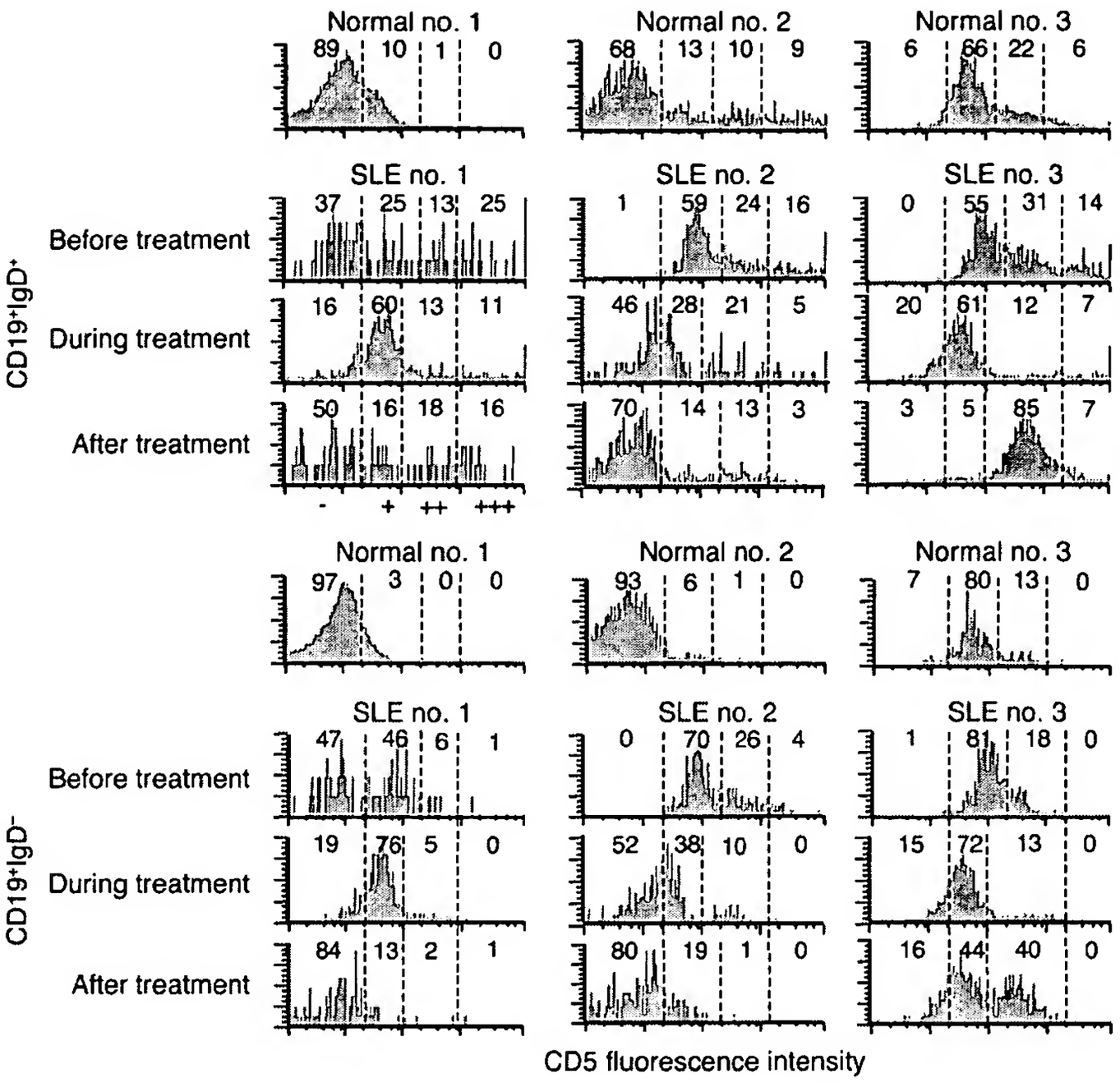

Figure 6
CD5 subsets of peripheral B cells diminish following treatment with humanized anti-CD154 Ab (h5c8). Freshly isolated PBMCs from active-SLE patients and normal volunteers were assessed for CD5 expression in B cell subsets by FACS analysis following staining with FITC-conjugated anti-IgD, PE-conjugated anti-CD19, and TC-conjugated anti-CD5. Histograms of CD5 expression on B cells from normal volunteers or active-SLE patients before treatment, during treatment (SLE nos. 1 and 2, 2 months; SLE no. 3, 1 month), or after withdrawal of treatment (SLE no. 1, 3 months after; SLE no. 2, 20 months after; SLE no. 3, 2 months after) are shown.

the pretreatment score; $P < 0.05$). Moreover, in the three patients (SLE nos. 1, 2, and 4) examined 20 months after the final treatment, the mean SLEDAI was 2.7 ± 2.5 (17% ± 14%), compared with a mean score of 12.7 ± 4.1 in these patients before treatment ($P < 0.05$). The major determinants of the improvement in SLEDAI were proteinuria, changes in anti-dsDNA Ab, and improvement in urinary-sediment and complement levels. Notably, all clinical improvements occurred in the setting of a lowered daily dose of prednisone from a median of 22.5 mg/d (range 5–30 mg/d) at base line to a median of 12.5 mg/d (range 3.75–20 mg/d) at 1 month post-treatment.

The clinical course of the four active-SLE patients treated with humanized anti-CD154 mAb (BG9588, 5c8) showed two different patterns following treatment withdrawal. SLE nos. 1 and 2 continued to improve in all aspects of their disease with the exception of a temporary increase in anti-dsDNA Ab levels in SLE no. 1 at 6 months after treatment was withdrawn. Importantly, SLE nos. 1 and 2 achieved and maintained complete renal remission without any additional clinical intervention following treatment withdrawal. SLE nos. 3 and 4 exhibited a different pattern. Although both SLE nos. 3 and 4 improved during treatment with humanized anti-CD154 mAb (BG9588, 5c8) and immediately following treatment withdrawal, they eventually exhibited disease reactivation and required additional immunosuppressive therapy.

Discussion

Although the results reported here were derived from an uncontrolled open-label study involving a small number of patients, the data suggest a central role of CD154-CD40 interactions in SLE, since specifically blocking this receptor-ligand pair in vivo significantly reduced serum autoantibodies, proteinuria, and an index of disease activity, SLEDAI. Importantly, anti-CD154 treatment decreased circulating $CD38^{bright}$, Ig-secreting cells in a time course that was generally similar to that of the reduction of serum anti-dsDNA Ab. These results were similar to the loss of Ig- and anti-DNA–secreting cells, as detected by ELISpot, that was previously reported following treatment of SLE patients with the humanized anti-CD154 BG9588 mAb (40). In addition, blocking the CD154-CD40 pair during in vitro cultures of highly purified B cells or during in vivo treatment of active-SLE patients reduced spontaneous B cell proliferation as well as the presence of $CD38^{positive}$ B cells ($CD38^{+/++}IgD^{+}$ or $CD38^{+}IgD^{-}$), which have previously been described in both tonsil tissue from non-autoimmune healthy controls (5) and SLE patients (22) as intermediates in the pathway to Ig-secreting cell development. Moreover, treatment with anti-CD154 decreased the appearance of pre-switch IgD^{+} B cells that were bright for CD5 or CD27 in the periphery with a time course similar to that of the disappearance of $CD38^{+/++}$ Ig-secreting cell differentiation intermediates or Ig-secreting cells. Finally, B cells that spontaneously expressed CD5 or CD27 at a low

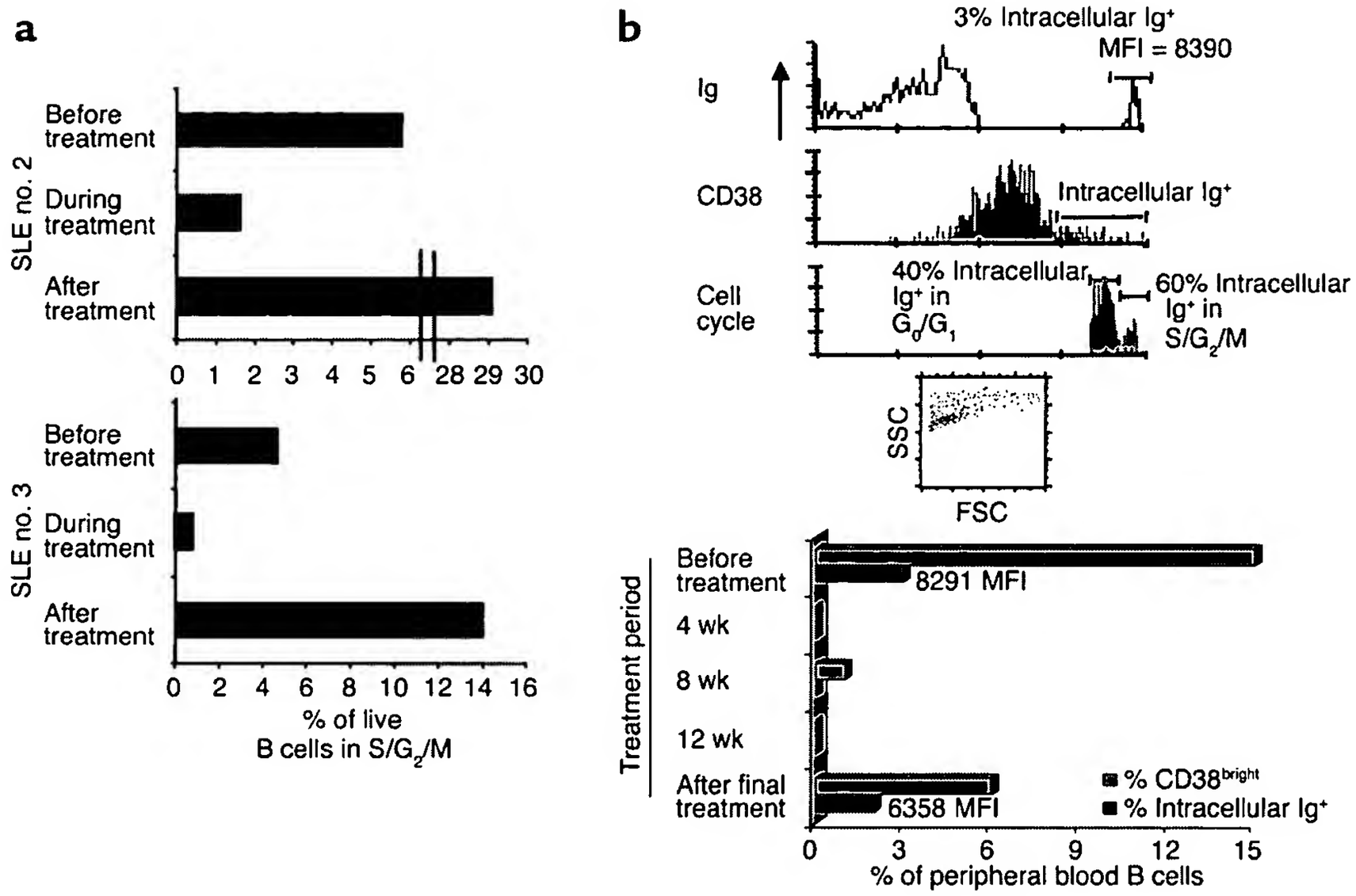

Figure 7

Anti-CD154 treatment eliminates proliferating B cells and Ig-secreting B cells from the periphery of active-SLE patients. (**a**) Freshly isolated PBMCs from active-SLE patients before treatment, after two treatments, and 2–3 months after the final treatment were assessed for cell cycle following permeabilization, fixation, and staining with propidium iodide and APC-conjugated anti-CD19 Ab. The percentages of live cells in the S, G_2, or M stage are depicted. (**b**) Freshly isolated PBMCs from SLE no. 3 before treatment, after two treatments, and 3 months after the final treatment were assessed for intracellular Ig and cell cycle following permeabilization, fixation, and staining with propidium iodide and FITC-conjugated anti-Ig and APC-conjugated anti-CD19 Ab. The presence of IC Ig, the expression of CD38, and cell cycle status are depicted. The percentage of IC Ig^+ B cells was determined by subtraction of the histogram generated for nonpermeabilized cells (surface Ig–positive) from the one generated for permeabilized cells (surface and IC Ig–positive) using CellQuest. The percentages of live cells that are $CD38^{bright}$ or IC Ig^+ are depicted. SSC, side scatter; FSC, forward scatter.

level, or the early-activation antigens CD69 and CD154, disappeared from the periphery of SLE patients during the post-treatment period. Together, these results suggest that spontaneous CD154-CD40 interactions in active-SLE patients drive B cell activation, proliferation, and differentiation to autoantibody-secreting plasma cells that mediate proteinuria and disease activity. Since CD154-CD40 interactions are essential for the development of GC reactions (11), and the Ig-secreting cells found in active-SLE patients have the mutational patterns characteristic of plasma cells generated during GC reactions (42–46), the data are consistent with the conclusion that GC reactions play a central role in the systemic B cell overactivity that is characteristic of SLE.

Previous studies have defined B cell subsets from inflamed secondary lymphoid tissue such as tonsil (5, 15–19) or the periphery of active-SLE patients (22–26) by expression of IgD and CD38. Of note, the current results confirm published studies that have demonstrated that B cells in the periphery of active-SLE patients express significantly higher levels of CD38 than B cells in the periphery of normal, non-autoimmune individuals (22, 25, 26). In tonsil, pre-switch $IgD^+CD38^{+/++}$ cells have been defined as activated naive, follicular, mantle zone, or GC founder cells (5, 15, 20, 21) and, when isolated from inflamed tonsils, spontaneously differentiate to $CD38^{+++}IgD^+$ Ig-secreting cells in vitro (data not shown). Post-switch $CD38^{+/++}IgD^-$ B cells are known as GC cells and, when isolated from inflamed tonsils, spontaneously differentiate in vitro to either $CD38^-IgD^-$ memory cells (5) or $CD38^{+++}IgD^-$ Ig-secreting cells (data not shown). Importantly, $CD38^{-/+/++}$ B cells isolated from the periphery of active-SLE patients have been observed to differentiate spontaneously to Ig-secreting cells when cultured in vitro (22), confirming the role of $CD38^{-/+/++}$ cells in the periphery of SLE patients as Ig-secreting differentiation intermediates. Of note, the percentages of $CD38^{+/++}IgD^+$ Ig-secreting cell differentiation intermediates and of $CD38^{+++}IgD^+$ Ig-secreting cell populations were significantly greater in the blood of active-SLE patients than in the periphery of normal non-autoimmune volunteers, whereas the percentage of $CD38^-IgD^+$ peripheral B cells was equivalent in active-SLE patients and normal non-autoimmune volunteers. These data suggest that the presence of $CD38^{+/++}$ pre-switch IgD^+ B cells in the periphery of SLE patients is associated with active disease. There were no significant differences between normals and active-SLE patients in the post-switch IgD^- subsets

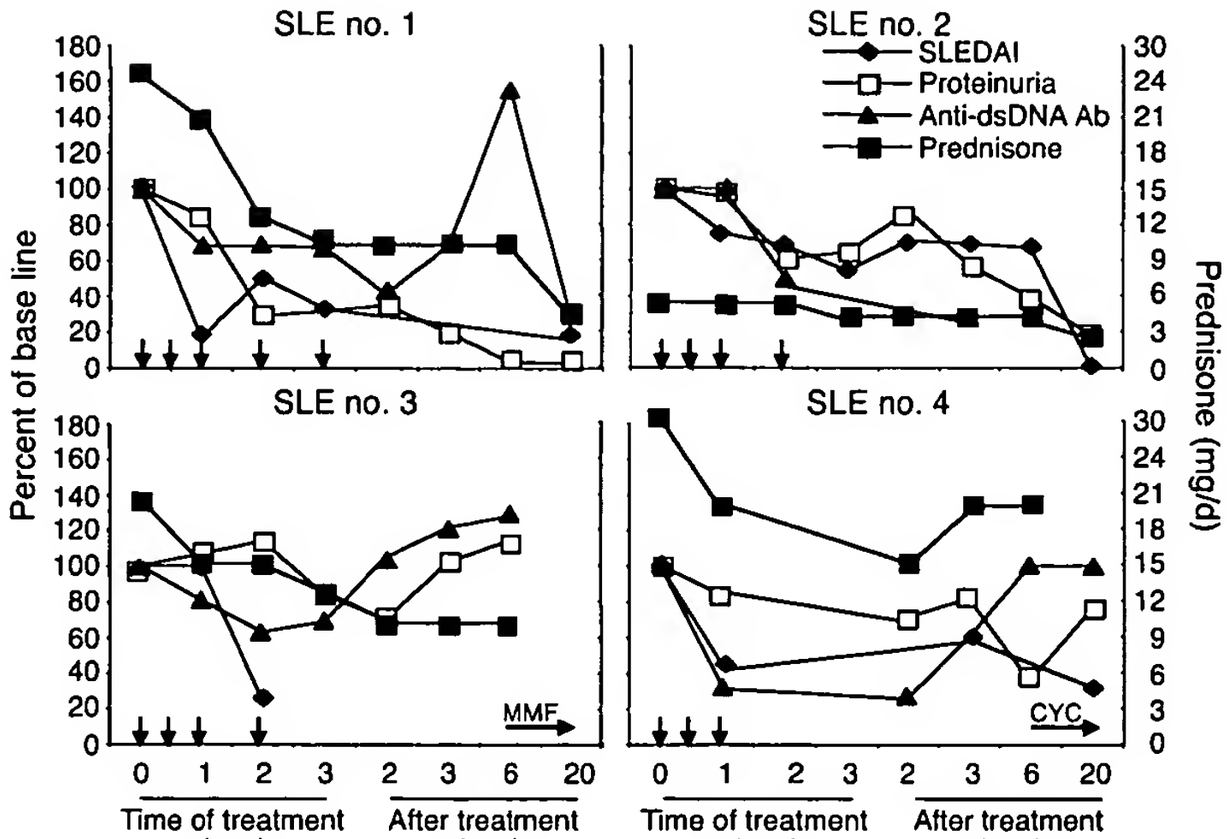

Figure 8
Treatment of active-SLE patients with humanized anti-CD154 mAb (BG9588, 5c8) decreases anti-DNA Ab levels and the degree of proteinuria and improves SLEDAI. Changes in anti-dsDNA Ab levels, 24-hour urinary protein excretion (proteinuria), and SLEDAI are expressed on the left axis as percentage of base-line levels. Prednisone dose is shown on the right axis in mg/d. Vertical arrows indicate infusions of humanized anti-CD154 mAb (BG9588, 5c8). Horizontal arrows indicate additional immunosuppressive therapy. MMF, mycophenolate mofetil; CYC, cyclophosphamide.

defined by CD38. These data extend published observations regarding $CD38^{positive}$, GC-derived B cells found in the circulation of active-SLE patients by examining the role of CD154-CD40 interactions in their generation and maintenance, especially the $CD38^{+++}$ Ig-secreting subsets. It is interesting to speculate whether the Ig-secreting cells in the periphery of active-SLE patients migrate to the kidney, as has been shown for lupus-prone mice (47).

In the present study, evaluation of B cells expressing CD38 in the periphery of active-SLE patients before, during, and after treatment with anti-CD154 mAb extends earlier observations by demonstrating that the $CD38^{+/++}$ intermediates in the differentiation path to Ig-secreting cells and $CD38^{+++}$ Ig-secreting cells in the periphery of active-SLE patients disappear from the circulation following one to two treatments with anti-CD154 and reappear in the periphery following treatment withdrawal. These results extend previously published data that define $CD38^{+/++}$ in the periphery of SLE patients as a differentiation intermediate in the path to Ig-secreting cells (22), in that they indicate that the presence of peripheral $CD38^{+/++}$ Ig-secreting cell differentiation intermediates and $CD38^{+++}$ Ig-secreting cells in the peripheral blood of active-SLE patients is dependent on CD154-CD40 interactions. Importantly, the upregulation of CD38 expression upon ligation of CD40 on B cells has been demonstrated at both the mRNA (6) and the protein (7) level. The present observations should be considered in light of previous observations indicating that maintenance of follicular/GC reactions requires ongoing signaling through CD154-CD40 interactions. Specifically, entire pre-switch follicles and post-switch GCs rapidly disassemble following administration of an anti-CD154 mAb to an immunized non-autoimmune mouse (30) or lupus-prone mouse strains with spontaneous follicular/GC reactions (31, 32). Moreover, blocking CD154-CD40 interactions in vivo abolished antigen-stimulated clonal expansion of B cells in GCs (33, 34) and decreased the development of memory B cells (5, 35). Furthermore, CD40 ligation preferentially induced differentiation of $CD38^{++}IgD^{-}$ and $CD38^{+}IgD^{-}$ GC B cells to memory cells defined by phenotype as well as function (36). In conclusion, the abundance of $CD38^{+/++}$ Ig-secreting cell differentiation intermediates and $CD38^{+++}$ Ig-secreting cells in the peripheral blood of active-SLE patients is likely to be dependent on overactive GC reactions that are themselves dependent on the CD154-CD40 ligand-receptor pair. Of note, although the majority of the IgD^{-} B cells in the active-SLE patients enrolled in the anti-CD154 Ab trial expressed surface IgM, the isotype of $CD38^{+++}IgD^{-}$ plasma cells cannot be determined by surface staining, since plasma cells are largely sIg^{-}.

Memory and Ig-secreting B cell subsets have been defined in inflamed secondary lymphoid tissue and in the periphery of active-SLE patients by differential expression of CD27 and CD5. In inflamed secondary lymphoid tissue, memory and Ig-secreting B cell subsets have been identified in the subepithelial regions of tonsil. Of note, memory cells are Ig-secreting cell differentiation intermediates that can be driven to Ig-secreting cells following engagement of CD40 (37, 38). IC Ig^{+} $IgM^{+}IgD^{+}$ B cells have been observed to express a higher level of CD27 and CD5 (13, 14, 18) when compared with IC Ig^{-} $IgM^{+}IgD^{+}$ B cells that have been defined as memory cells based on the presence of somatic hypermutation of Ig genes (13). Of note, differential CD27 expression has been used to distinguish IC Ig^{+} cells from IC Ig^{-} memory B cells isolated from the periphery of active-SLE patients (25, 27). In addition, somatic hypermutation of Ig is more extensive in the IC Ig^{+} population from either tonsil or the periphery of active-SLE patients than in the IC Ig^{-} memory population (13, 27). By contrast, the CD27-negative population did not show evidence of Ig somatic hypermutation in B cells from tonsil or the periphery of active-SLE patients. Studies using tonsillar B cells have shown that a high level of CD5 marks subepithelial plasmablasts and

a low level of CD5 identifies activated pre- or post-switch B cells in the follicular/GC or extrafollicular/subepithelial regions (14, 19). In vitro experiments have demonstrated that CD5 can be induced on the surface of B cells following stimulation through CD40 (9) or following coculture with anti-CD3–activated T cells (10). Although studies have demonstrated that Ig-secreting cells can be generated from either $CD5^-$ or $CD5^{positive}$ B cells (10), plasmablasts that secrete anti-dsDNA Ab express a high level of CD5, and fully differentiated, nonproliferating plasma cells have been observed to be CD5-negative (29, 48). In the current study, the percentage and MFI of CD27 and CD5 expressed on the surface of pre-switch B cells from the periphery of SLE patients were significantly higher than those expressed on peripheral or tonsillar B cells from non-autoimmune controls. In addition, the dependence of these pre-switch IgD^+ subsets on ongoing CD154-CD40 interactions was demonstrated by the finding that treatment of active-SLE patients with anti-CD154 mAb decreased the percentage of $CD5^{bright}$, $CD27^{bright}$, and IC Ig^+ $CD38^{bright}$ B cells in the periphery. These data confirm that CD154-CD40 interactions occurring in active-SLE patients generate Ig-secreting cell differentiation intermediates such as CD27-positive memory cells and Ig-secreting cells that can be identified by a number of markers including high expression of CD38, CD27, and CD5.

In normal individuals as well as autoimmune individuals, targeted somatic hypermutation of Ig has been found in CD27-positive but not CD27-negative pre-switch IgD^+ B cells (13, 25, 27). Moreover, histological GCs (49) and a hallmark of GC reactions, targeted RGYW/WRCY somatic hypermutation of heavy and light chain Ig genes, have been demonstrated to be absent in X-linked hyper-IgM patients genetically deficient in CD154 expression (50, 51) and thus functional CD154-CD40 interactions. Interestingly, low percentages of $CD27^+IgD^+$ peripheral B cells have been reported in X-linked hyper-IgM patients with a complete block of functional CD154-CD40 interactions (the mean of the values from the two reports is 4.4% ± 1.3%, range 1–12%, $n = 8$) (52, 53). Of note, the percentage of CD27-positive IgD^+ B cells in the periphery of X-linked hyper-IgM patients is markedly lower ($P < 0.05$) than that found in the periphery of normal non-autoimmune volunteers (36.2% ± 6.9%; Table 2), which in turn is significantly lower than that found in the active-SLE patients before treatment with humanized anti-CD154 mAb (97.0% ± 2.1%; Table 2). In light of these observations, signals other than CD154-CD40 interactions may make a minor contribution to the appearance of $CD27^+IgD^+$ B cells in the periphery, as has been observed in some circumstances for expression of other activation/differentiation antigens such as CD69, CD154, and CD5 (5, 9, 11). This contribution is most apparent in patients that lack functional CD154-CD40 interactions, such as X-linked hyper-IgM, since the percentage of $CD27^+$ circulating IgD^+ peripheral B cells is small. In normal individuals, however, the vast majority of $CD27^+IgD^+$ B cells appear to be pre-switch memory cells that have originated from GCs in secondary lymphoid tissues.

Active-SLE patients have a higher percentage of circulating B cells expressing early-activation antigens such as CD69, CD154, or a low level of CD5 than do non-autoimmune individuals. Treatment of active-SLE patients with anti-CD154 mAb resulted in a decrease in B cells expressing CD69, CD154, or a low level of CD5 during the post-treatment time period. Of note, although ligation of CD40 on highly purified B cells from normal individuals during in vitro culture induced expression of CD5 (9) and CD154 (5), there are other signals that also contribute to increased expression of these early-activation antigens, including cytokines and engagement of sIg (11), and these other signals may lead to continued expression following blockade of CD154-CD40 interactions. In this regard, the initial stages of B cell activation have been previously shown to be independent of CD154-CD40 interactions (54, 55). The finding that markers of early B cell activation such as CD69 and CD154 continue to be expressed on B cells from the periphery of active-SLE patients after treatment with anti-CD154 mAb suggests that the eventual decrease in these early-activation markers may be secondary to improvement in disease activity of SLE and not a primary effect of blocking CD154-CD40 interactions.

Treatment of active-SLE patients with anti-CD154 mAb led to a rapid decrease in serum anti-dsDNA autoantibody levels that paralleled the disappearance of $CD38^{bright}$ IC Ig^+ plasma cells from the circulation. Of note, Ig-secreting cells producing anti-dsDNA Ab have been previously shown to be dependent on ongoing proliferation, since anti-dsDNA Ab levels, but not levels of other autoantibodies, can be inhibited by treatment with antiproliferative drugs such as cyclophosphamide (56–60). These data confirm in vitro experiments that demonstrated that CD154 expression on B cells leads to homotypic CD154-CD40 interactions that mediate proliferation, Ig secretion, and a positive feedback loop that results in increased CD154 expression on B cells (5, 56–64), as well as in vivo experiments that demonstrated that mice transgenic for CD154 on all cells (65, 66), or B cells alone (67), spontaneously developed plasmablasts that secreted pathogenic anti-dsDNA Ab.

In summary, the current data demonstrate that treating active-SLE nephritis patients with an mAb against CD154 inhibits disease activity as well as abnormal B cell differentiation that leads to the presence of circulating Ig-secreting cells and serum anti-dsDNA Ab (39). Of note, anti-cardiolipin levels, anti-nuclear antigen (ANA) titer, and total serum Ig levels did not change significantly with treatment (data not shown) (40). Flow cytometric analysis of peripheral B cell subsets in active-SLE patients, defined by IgD, CD38, cell cycle status, and the presence of IC Ig, demonstrated that the disappearance of Ig-secreting cells in the periphery was associated with decreases in anti-dsDNA Ab levels, proteinuria, and SLEDAI. Together, these observations indicate that in vivo CD154-CD40 interactions drive

SLE disease activity and aberrations in the peripheral B cell compartment of active-SLE patients.

Although this hypothesis would be stronger following testing in a larger, properly controlled clinical trial, the findings are consistent with published preclinical work and suggest that CD154-CD40 interactions should be a central target of therapy in SLE. Moreover, the association between normalization of B cell subsets and clinical improvements in disease activity, in addition to the complete remission of the two patients treated with the longest course of humanized anti-CD154 mAb, strongly suggests the benefit of further exploring this treatment in active SLE. Since anti-CD154 treatment was associated with an increased frequency of thrombotic events in treated patients, the goal would be to develop a safer means to test the hypothesis generated by these and previous studies. The mechanism of thrombosis is not understood, although CD154-CD40 interactions involving activated platelets (68–73) and endothelial cells (74–89) in persons with underlying vascular disease may play a significant role. The use of another mAb to CD154 (24-31; IDEC-131) in SLE was apparently not associated with an increased frequency of thrombosis, although minimal efficacy was also noted (90, 91). To find a safe and effective means to block CD154-CD40 interactions in active-SLE patients remains a challenge.

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